

Physiology of  
THE DOMESTIC FOWL

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*British Egg Marketing Board Symposium  
Number One*

# Physiology of the DOMESTIC FOWL

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*Edited by*

C. HORTON-SMITH

AND

E. C. AMOROSO

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## PREFACE

THIS VOLUME records the proceedings of a Symposium organised by the Scientific Advisory Committee of the British Egg Marketing Board and held in the University of Nottingham, School of Agriculture, Sutton Bonington, 16th-18th December 1964. The Committee came into being in April 1961 as a result of a report by Professor A. C. Chibnall who had been asked to advise the Board on what might be done to promote research relevant to egg production in domestic fowl. The Committee's terms of reference were as follows:

To promote the scientific study of domestic fowl by drawing attention to its importance, by stimulating the recruitment and training of research workers, by organising basic and applied research in poultry husbandry and in the physiology, pathology, genetics, nutrition and biochemistry of fowl, and by facilitating communication between those working in this field.

In the course of four and a half years the Committee has made numerous recommendations to the Board, all of which have been accepted. In facilitating communication, in accordance with its final instruction, the Committee obtained support for the journal, *British Poultry Science*, and published a directory of scientists and institutions concerned with poultry research. The organisation of the 1964 Symposium as the first of what it is hoped will be an annual series represented, however, the major step in this direction. The second Symposium took place on 22nd and 23rd September 1965, again at Sutton Bonington, this time on *Protein Utilisation by Poultry*. The proceedings of this second Symposium will be published similarly in book form as soon as possible.

The aims of the Symposia are to enable those working in the field to meet each other, to promote the exchange of information and to provide an opportunity for reviewing the current state of knowledge in particular aspects of poultry science. It is hoped further that the Symposia will indicate where further effort could most fruitfully be concentrated. The 1964 Symposium was very well attended and all those who were present will agree that it accomplished all these aims and set a high standard which we have every expectation will be maintained in subsequent ones.

It was my pleasant duty as Chairman of the first session of the 1964 Symposium to welcome all participants on behalf of the British Egg Marketing Board and particularly the overseas visitors, notably



## PREFACE

Professor Nalbandov, Dr Lorenz and Dr Romijn whose presence contributed much to the success of the meeting.

The Scientific Advisory Committee offers its warmest thanks to all who took an active part in the meeting especially to those who prepared manuscripts for publication. Grateful acknowledgement is also made to Dr C. Horton-Smith, who with the assistance of Professor Amoroso, on the biological side, undertook the not inconsiderable editorial duties involved in the publication of this volume. Best thanks are also due to Dr Norman Knowles, Secretary of the Committee, who was largely responsible for the organisation of the meeting.

A. S. P.

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# CONTENTS

PREFACE	... ..	v
MEMBERSHIP OF THE SCIENTIFIC ADVISORY		
COMMITTEE	... ..	vi

## PART I

### REPRODUCTIVE PHYSIOLOGY AND ENDOCRINOLOGY

1.	HORMONE CONTROL OF OVULATION ... ..	3
	<i>D. M. Nelson &amp; A. V. Nalbandov</i>	
2.	ASPECTS OF THE PHYSIOLOGY OF THE TRANSPORT OF THE OVUM THROUGH THE OVIDUCT OF THE DOMESTIC HEN ... ..	11
	<i>A. B. Gilbert, P. E. Lake &amp; D. G. M. Wood-Gush</i>	
3.	EFFECT OF FOWL GONADOTROPHIN FOLLOWING TREATMENT WITH A PITUITARY INHIBITOR ... ..	14
	<i>Margaret E. Mitchell</i>	
4.	OESTROGENS IN PLASMA FROM THE DOMESTIC FOWL ...	23
	<i>J. E. O'Grady</i>	
5.	SOME OBSERVATIONS ON THE FINE STRUCTURE AND HISTOCHEMISTRY OF THE OVARIAN FOLLICLE OF THE FOWL ... ..	30
	<i>G. M. Wyburn &amp; A. H. Baillie</i>	
6.	BEHAVIOUR OF SPERMATOZOA IN THE OVIDUCT IN RELATION TO FERTILITY ... ..	39
	<i>F. W. Lorenz</i>	
7.	THE ORIGIN AND COMPOSITION OF FOWL SEMEN ...	44
	<i>P. E. Lake &amp; M. H. El Jack</i>	
8.	FERTILITY IN THE MALE IN RELATION TO NATURAL AND ARTIFICIAL INSEMINATION ... ..	52
	<i>M. Perch</i>	

## PART II

## METABOLISM AND NUTRITION

9. THE ACCUMULATION OF WATER AND ELECTROLYTES  
IN THE EGG OF THE HEN ... .. 63  
*M. H. Draper*
10. RECOVERY OF YOLK PIGMENT IN THE LAYING FOWL ... 75  
*H. S. Bayley & Gillian M. Newlands*
11. SOME ASPECTS OF THE LIPID METABOLISM OF THE  
CHICK EMBRYO ... .. 87  
*R. C. Noble & J. H. Moore*
12. SOME EFFECTS OF ADRENOCORTICOTROPHIC HORMONE  
ON BURSECTOMISED AND INTACT CHICKENS ... .. 103  
*B. M. Freeman, L. G. Chubb & A. W. Pearson*
13. THE EFFECTS OF GLUCAGON AND INSULIN ON THE  
PLASMA GLUCOSE AND UNESTERIFIED FATTY  
ACIDS OF THE DOMESTIC FOWL ... .. 113  
*P. J. Heald*
14. THE MECHANISM OF PROTEIN SYNTHESIS IN THE HEN  
OVIDUCT ... .. 125  
*N. H. Carey*
15. CHANGES IN THE WATER-SOLUBLE PROTEINS OF THE  
AVIAN OVIDUCT IN RELATION TO REPRODUCTION  
AND FOLIC ACID DEFICIENCY ... .. 133  
*W. O. Brown*
16. PROTEIN DIGESTION AND METABOLISM IN THE  
COLOSTOMISED LAYING HEN ... .. 146  
*E. Sqaunce*
17. AMINO ACID INTERACTIONS IN POULTRY NUTRITION ... 155  
*D. Lewis*
18. AMINO ACID ALLOWANCES FOR LAYERS ... .. 163  
*B. R. Taylor, C. G. Payne & D. Lewis*
19. BIOASSAY OF AMINO ACIDS ... .. 171  
*H. O. Uwaegbute & D. Lewis*
20. CURRENT VIEWS ON THE ROLE OF THE GUT FLORA IN  
NUTRITION OF THE CHICKEN ... .. 181  
*M. E. Coates & D. J. Jayne-Williams*

## PART III

### CALCIUM METABOLISM AND EGGSHELL FORMATION

- |     |   |     |
|-----|---|-----|
| 21. | THE FUNCTIONAL ANATOMY OF THE AVIAN SHELL<br>GLAND ... ..         | 191 |
|     | <i>R. D. Hodges</i>   |     |
| 22. | THE ENDOCRINE CONTROL OF CALCIUM METABOLISM<br>IN THE FOWL ... .. | 199 |
|     | <i>T. G. Taylor</i>   |     |
| 23. | SHELL STRENGTH ... ..   | 203 |
|     | <i>C. Tyler</i>   |     |

## PART IV

### ENVIRONMENTAL PHYSIOLOGY

- |     |   |     |
|-----|---|-----|
| 24. | HEAT REGULATION AND ENERGY METABOLISM IN THE<br>DOMESTIC FOWL ... ..  | 211 |
|     | <i>C. Romijn &amp; W. Lokhorst</i>  |     |
| 25. | THE RESPONSE OF THE IMMATURE CHICKEN TO<br>AMBIENT TEMPERATURE ... ..                                       | 228 |
|     | <i>G. W. Osbaldiston</i>  |     |
| 26. | ENVIRONMENTAL TEMPERATURE AND EGG<br>PRODUCTION ... ..  | 235 |
|     | <i>C. G. Payne</i>  |     |
| 27. | CONTROLLED ENVIRONMENTAL HOUSING: THE<br>PRACTICAL PROBLEM OF OBTAINING THE<br>PHYSIOLOGICAL OPTIMUM ... .. | 242 |
|     | <i>D. W. Sainsbury</i>  |     |

## PART V

### PHARMACOLOGY AND GENERAL PHYSIOLOGY

- |     |  |     |
|-----|--|-----|
| 28. | ANTICHOLINESTERASE DRUGS IN THE FOWL ... ..  | 249 |
|     | <i>W. C. Bouman</i>  |     |
| 29. | PHARMACOLOGICAL RESPONSES OF THE ISOLATED<br>OESOPHAGUS AND CROP OF THE CHICK ... .. | 261 |
|     | <i>Sally D. Everett</i>  |     |

# CONTENTS

5.	PHARMACOLOGICAL RESPONSES OF THE SMOOTH MUSCLE OF THE CHICK AMNION ... ..	274
	<i>A. W. Guthbert</i>	
1.	CATECHOLAMINES IN THE CHICK ... ..	279
	<i>B. A. Callingham &amp; Rosemary Cass</i>	
12.	RENAL FUNCTION IN THE FOWL ... ..	286
	<i>A. H. Sykes</i>	
13.	LIVER BLOOD FLOW IN THE TURKEY ... ..	294
	<i>M. J. Clarkson &amp; T. G. Richards</i>	
34.	AFFERENT PATHWAYS IN THE VAGUS AND THEIR INFLUENCE ON AVIAN BREATHING: A REVIEW ... ..	302
	<i>A. S. King</i>	
	LIST OF PARTICIPANTS ... ..	311
	INDEX OF NAMES ... ..	317
	INDEX OF SUBJECTS ... ..	324

PART I

REPRODUCTIVE PHYSIOLOGY AND  
ENDOCRINOLOGY

30.	PHARMACOLOGICAL RESPONSES OF THE SMOOTH MUSCLE OF THE CHICK AMNION ... ..	274
	<i>A. W. Cuthbert</i>	
31.	CATECHOLAMINES IN THE CHICK ... ..	279
	<i>B. A. Callingham &amp; Rosemary Cass</i>	
32.	RENAL FUNCTION IN THE FOWL ... ..	286
	<i>A. H. Sykes</i>	
33.	LIVER BLOOD FLOW IN THE TURKEY ... ..	294
	<i>M. J. Clarkson &amp; T. G. Richards</i>	
34.	AFFERENT PATHWAYS IN THE VAGUS AND THEIR INFLUENCE ON AVIAN BREATHING: A REVIEW ... ..	302
	<i>A. S. King</i>	
	LIST OF PARTICIPANTS ... ..	311
	INDEX OF NAMES ... ..	317
	INDEX OF SUBJECTS ... ..	324

PART I

REPRODUCTIVE PHYSIOLOGY AND  
ENDOCRINOLOGY



## HORMONE CONTROL OF OVULATION

D. M. NELSON\* AND A. V. NALBANDOV

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Urbana, Illinois, U.S.A.**Synopsis*

Using the OAAD method, the LH concentrations in the plasma and in the hypophyses of 80 laying hens were estimated. Three important peaks of LH release into the plasma are noted. The two peaks occurring respectively 13 and 8 hr. prior to ovulation are both thought to be essential in inducing rupture of the follicle. While both releases are thought to be mediated by the hypothalamus, the signal for the release at 13 hr. is thought to be neural in nature while the signal for the release 8 hr. prior to ovulation is thought to be caused by a hormone, perhaps progesterone. The first LH release during the laying cycle occurs 5 hr. after oviposition or 19 hr. prior to ovulation of the next ovum. This peak of LH release is probably not related to ovulation, but it is noted that it occurs at the time when the egg leaves the magnum and enters the uterus. This study is considered as a step towards an understanding of the hormonal mechanism controlling ovulation in the hen. The fact that LH can be reliably detected in plasma and pituitary tissue of laying hens (and cockerels), should greatly facilitate future research on problems of avian reproduction.

*Introduction*

Systematic study of the avian endocrine control systems is not very old. Among the oldest attempts to understand the role of the pituitary gland in reproduction are those of Hill, Corkill and Parkes (1934) and Hill and Parkes (1934), who were the first to hypophysectomise chickens. Subsequently, much additional work has appeared in print which clearly established that in the bird the gonads do indeed depend for support on hypophysial secretion. This has been amply confirmed both by experiments involving ablation of the adenohypophysis and those in which substitution therapy was used in either intact or hypophysectomised hens (Opel and Nalbandov, 1961a, b, c). None of the experimental work sheds much light on the mechanism controlling the

\* The experimental data presented here were collected by Mr D. M. Nelson in partial fulfilment of the requirements for a Ph.D. degree of the University of Illinois

cyclic function of the ovary of the laying hen resulting in the well-known clutch sequences which are typical of all avian species. Several attempts to explain the underlying control systems have been made, but none of them was satisfactory, primarily because they were based on conjectures rather than endocrine facts (for details, see Fraps, 1961). It appeared necessary to gather knowledge of the changes in hypophyseal hormone concentrations throughout the laying cycle. With the advent of the ovarian ascorbic acid depletion (OAAD) method, a tool became available which allowed the assay of individual glands for luteinising hormone (LH) content and concentration. The method also held out hope that LH concentrations in small quantities of plasma, obtained from living hens, some of which could then continue to complete the laying cycle, could be determined and perhaps correlated with the rate of synthesis and release of hypophyseal LH. Because the OAAD method is time consuming and cumbersome and because of the difficulty of obtaining adequate numbers of suitable donor animals, it was decided to concentrate on changes in LH concentration and to leave estimates of levels of FSH for later. It is the intent of the present paper to discuss the changes in plasma and hypophyseal LH concentration throughout one laying cycle of eighty laying Single Comb White Leghorn hens.

### *Materials and Methods*

The OAAD method used was the standard method proposed by Parlow (1961) which was somewhat modified for our purposes. The plasma was obtained by heart puncture from laying hens whose habitual laying cycles had been established during a pre-experimental period. In the experiments to be discussed here, blood was taken at different times following the oviposition of the  $C_1$  ovum (first ovulation of a clutch), and prior to ovulation of the  $C_3$  follicle. Following bleeding, the hens were killed and the adeno-hypophyses recovered for bioassay; the oviducts were inspected for the presence of eggs and their relative positions in the oviduct were recorded. In this experimental design, plasma and glands were available for each hour of the entire cycle, albeit from different hens. In one experiment hens were bled and their pituitary glands recovered following oviposition of the terminal egg of the clutch. In these hens no ovulation was expected to occur for 16 hr. after the lay of the terminal egg of the clutch.

### *Results and Discussion*

Before discussing the results obtained in the changes in LH during the laying cycle, a few words in support of the validity of the assay method are in order. The question was asked whether the method is capable of detecting LH in chicken plasma. Two different approaches to this problem provided reasonable assurance that LH is indeed

measurable in plasma. In the first approach it was reasoned that some estimate of the reliability of the method could be obtained if one were to introduce exogenous LH into a bird and ask the question how much of this LH could be demonstrated in the plasma by the OAAD method. Accordingly, 200  $\mu\text{g.}$  of an NIH-LH ( $B_1$ ) were injected intravenously into a male weighing 2,088 g. and blood was withdrawn by heart puncture from the bird at intervals up to 55 min. after the initial injection. It will be noted (Fig. 1) that there is a very significant rise in LH plasma concentration as compared to the pre-injection normal level. Parenthetically it should be noted that the half-life of such exogenous LH is exceedingly short and that by 10 min. after initial injection practically no exogenous LH is detected in the plasma. Fifteen min. after injection only the endogenous LH can be detected. This experiment was repeated several times in different animals with identical results. The second approach is perhaps more convincing. It was demonstrated in many animals that the plasma level of LH drops from a normal level of around 8-9  $\mu\text{g./100 ml.}$  to zero immediately following hypophysectomy of male chickens. For these reasons it appears highly probable that the assay does measure the endogenous LH in plasma or in pituitary tissue.

We can now proceed to examine the results obtained on the assays of plasma and hypophyses of laying hens during the cycle. The data are shown graphically in Fig. 2, in which the LH concentrations are expressed in terms of the NIH-LH used as a reference standard in each of the separate assays. The time intervals along the abscissa are arranged to show the actual intervals in hours from the *lay* of  $C_1$  ovum to sampling of fluids or tissue (top row), as well as the calculated intervals from sampling to the expected *ovulation* of the  $C_3$  ovum which, of course, was never ovulated because the hen was killed before that event. The  $C_2$  ovulation was being converted into an egg in the oviduct during the entire sampling period. Inspection of Fig. 2 will show that as far as plasma LH concentration is concerned there are 3 peaks of LH release which occur about 5, 12 and 18 hr. after oviposition of the  $C_1$ , or approximately 20, 12 and 8 hr. prior to ovulation of the  $C_3$ . For the time being, let us ignore the first peak of LH release and consider the 2 peaks which occur 8 and 12 hr. prior to ovulation of  $C_3$ . Both peaks are highly significant in their elevations over the base level and the conclusion appears justified that two LH releases occur prior to the shedding of the next egg.

This finding was completely unexpected and difficult to understand until we recalled that there are two previous independent estimates of the time of LH release available in the literature. The first estimate was made by Rothchild and Fraps (1949) who hypophysectomized hens which were laying regularly 10 to 2 hr. prior to expected ovulation. This experiment led to the conclusion that the release of LH occurs about 8 hr. prior to ovulation. Somewhat later Van Tienhoven

Nalbandov and Norton (1954) blocked ovulation in laying hens by the use of an adrenergic blocking agent, Dibenamine. They found that the most effective blockade was produced about 14 hr. prior to expected ovulation and suggested that the neural stimulus for LH release takes place at about that time. For many years the possible reasons for the discrepancy between these two estimates was an enigma. On the basis of the direct assay of the plasma LH concentration it now appears that

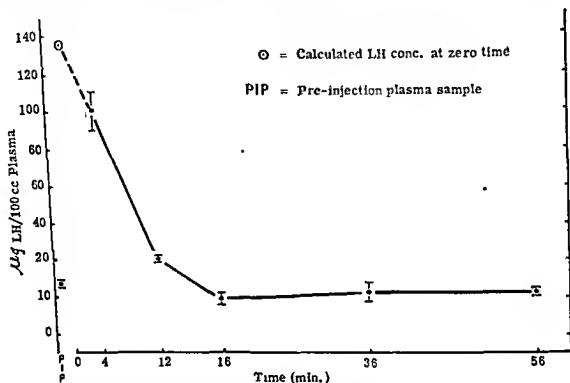


FIG. 1. Recovery of exogenous LH injected into intact cockerels. The levels of LH at 0 time and 18 minutes and after the intravenous injection of exogenous LH, represent endogenous LH.

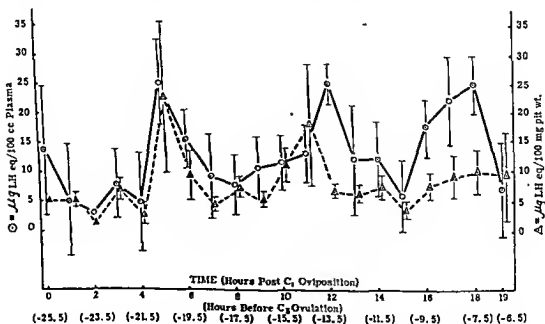


FIG. 2. Changes in LH concentration in plasma (solid line) and hypophyses of 80 laying hens. Abscissa shows hours after lay of  $C_1$  egg (upper row) and hours prior to ovulation of  $C_3$  ovum (bottom row).

both estimates were amazingly accurate and that there are indeed two LH releases. It remains to be understood why two releases are necessary.

It should be noted that the 14-hr. peak is the one that was blocked by Dibenzamine, which suggests that this is the release that is caused by a neural signal. The fact that Rothchild and Fraps (1949) and others were also able to cause LH release in hens by the injection of progesterone 6 to 8 hr. prior to ovulation further suggests that the LH release occurring at 8 hr. may be induced by a hormonal signal. These conjectures must await further experimental manipulations before they can be endowed with the stature of a working hypothesis.

Further inspection of the data in Fig. 2 shows another interesting fact. There is a good and significant correlation between hypophysial LH content and plasma concentration, but only for the first 2 peaks of release. This suggests that there is increased synthesis as well as release of LH for the first 2 peaks but that at the third peak the pituitary releases whatever hormone it has stored but does not synthesise new LH. The biological significance of this observation is difficult to evaluate in the absence of further experiments.

It was quite unexpected to find the first peak of LH synthesis and release. It is noted that it occurs 5 hr. after the ovulation of  $C_1$ . By that time the  $C_1$  ovum has passed through the magnum and isthmus and has entered the uterus. It may be recalled that Huston and Nalbandov (1953) have shown that the presence in the magnum of a thread (or of any other foreign body) prevents ovulation without impairing follicular development. Ovulation in such blocked hens can be induced at any time by either the injection of LH or of a large dose of progesterone. This was interpreted to mean the LH-release was blocked by neural signals originating in the magnum. It is curious to note that the first peak of LH release occurs shortly after the egg leaves the magnum. Since the thread presumably blocks all 3 peaks and since, as shown by the data here presented, LH is normally released when the egg enters the uterus, it seems reasonable to assume that this first peak has nothing to do with induction of ovulation but may provide the LH to cause the ovulatory spurt of follicular growth which is as characteristic of chickens as it is of mammals. From the Rothchild-Van Tienhoven data it appears reasonably certain that both the 14 and 8 hr. pre-ovulatory releases of LH are necessary for ovulation, but why a double release is required remains unknown. It will be interesting to see what happens to the follicle stimulating hormone (FSH) concentration in pituitaries and in the plasma (if technical limitations permit its estimation in the plasma) at the times when LH peaks are detected during the ovulatory cycle.

Of some interest are the fluctuations in the weights of the pituitary glands during the laying cycle. It is seen (Fig. 3) that, in general, the peaks in pituitary weight coincide with the peaks of LH release shown

in Fig. 2. The peaks at 22 and 8 hr. prior to ovulation are significantly different from the lowest values preceding and following them. None of the values between 22 and 12 hr. prior to ovulation is significantly different from the other. Taking the statistical significance of the weights at the different times into consideration, one can summarise the data on hypophysial weight changes as follows. There is an increase in pituitary weight from 22 to 21 hr. prior to ovulation. From 21 to 12 hr. the weight remains unchanged and high. After the LH release 12 hr. prior to ovulation, hypophysial weight drops drastically in 1 hr. The weight is gradually regained by 8 hr. and, after the LH release

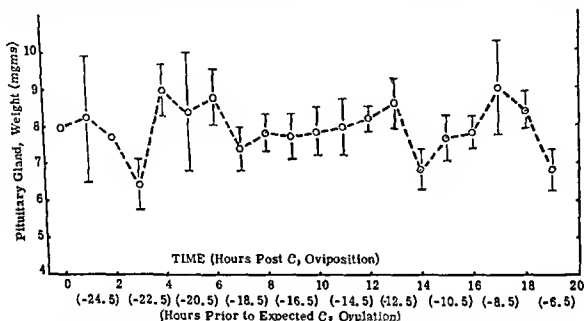


FIG. 3. Changes in pituitary weights throughout the laying cycle. These are the same glands which were assayed for LH content (Figure 2). Note that peaks in pituitary weight roughly correspond to peaks of LH release.

occurring at 8 hr., the weight drops again to base level. The biological significance of these changes in pituitary weight are unknown. Because of the magnitude of the changes in weight it is highly unlikely that an increase in weight is due to accumulation of hormones just as a drop in weight cannot be reasonably ascribed to release of LH into the blood stream. For the time being it is probably safest to note the amazingly close correlation between the hypophysial weight changes and the times of LH release from the pituitary gland.

There is one further point of interest which emerged from these studies. A comparison was made between the patterns of LH release into the blood stream and its hypophysial concentration in hens which had laid the terminal egg and those which were about to ovulate the second egg of the clutch. Hens laying 4- and 5-egg clutches were selected for this study and all of them were killed at the time of the 12 hr. pre-ovulatory LH release. It was intended to test the idea presented elsewhere (Nalbandov, 1958, 1961) that the clutch is terminated because the pituitary gland cannot synthesise enough hormone by the

time the terminal egg is laid and that a recovery period of about 24 hr. is required for the adenohypophysis to regain competence for adequate LH release. The results are summarised in Table 1. First, attention is called to the fact that no difference exists in the LH concentration in the pituitaries of the 8 hens which had laid the terminal egg of the

TABLE 1

*Comparison of LH concentrations in glands and plasma of hens having laid the terminal egg and those having laid the first egg of the clutch, sampled 12 hr. after oviposition*

Position in clutch	No. of hens	Pituitary wet weight (mg.)	LH concentration in		
			Pituitary $\mu\text{g./100 mg.}$	$\mu\text{g./whole}$ gland	Plasma $\mu\text{g./100 ml.}$
Terminal	8	$6.77 \pm 0.29^a$	$5.67 \pm 1.30$	$0.39 \pm 0.09$	$5.02 \pm 0.76$
Initial	12	$8.22 \pm 0.40^b$	$5.67 \pm 1.04$	$0.48 \pm 0.10$	$25.42 \pm 3.56^c$

<sup>a</sup> Means  $\pm$  standard error. <sup>b</sup>  $P = 0.05$ . <sup>c</sup>  $P < 0.001$ .

clutch and those of hens which had laid the  $C_1$  and had the  $C_2$  ovulation in the oviduct. Neither are the differences in  $\mu\text{g. LH/pituitary}$  or those between pituitary weights significant between these two groups. In contrast, the highly significant differences ( $P < 0.001$ ) in the plasma LH between the two groups is very striking. This clearly shows that the pituitary glands at the end of the clutch have as much LH reserve as they do at its beginning but that they release none or little of their hoard into the systemic circulation.

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# ASPECTS OF THE PHYSIOLOGY OF THE TRANSPORT OF THE OVUM THROUGH THE OVIDUCT OF THE DOMESTIC HEN

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## *Synopsis*

EGG-SHELL quality depends upon, among other things, the time which an egg spends in the shell gland prior to oviposition and the integrity of the calcium deposition process. Some experiments demonstrating physiological factors involved in the oviposition mechanism are described in this review.

## *Introduction*

Poor egg quality, in all its aspects, is a major concern of the poultry industry and malfunction of the oviduct secretory mechanisms or derangement of the movement of the egg down the oviduct may be involved. This review will be concerned only with physiological factors relating to poor egg-shell quality.

If calcium secretion is normal it would seem reasonable, in the present state of knowledge, to presume that the shorter the stay in the uterus the less satisfactory is the quantity of shell deposited (Burmester, 1940) and hence the quality of the egg-shell will be impaired. Thus the mechanisms involved in oviposition and its timing may well have a bearing on the general problem of egg-shell formation.

## *Effect of a foreign object in the oviduct on oviposition*

Experimentally it can be shown that surgical interference with the uterus can result in the oviposition of shell-less eggs. Huston and Nalbandov (1953) reported on the effects of placing a surgical thread in the oviduct on egg-laying rate and Sykes (1953) showed that if a thread was placed in the shell gland poorly shelled eggs were laid. The latter observation was confirmed by Lake and Gilbert (1964), and moreover the effect was found to be most marked when the thread was placed in the shell gland or utero-isthmus junction; in other regions there was little or no effect. There was no evidence that calcium

deposition was affected, but the results suggested that oviposition time was premature. It was found that oviposition times of shell-less eggs were always between the hours of 7 p.m. and 8 a.m. whereas normal eggs were laid between the hours of 7 a.m. and 7 p.m. In a study of nesting behaviour of the bird (Wood-Gush, 1963), soft-shelled eggs were always laid on the floor of the pen and the bird appeared to be completely unaware of this event. However, about 12 to 18 hr. subsequently, the bird did exhibit a perfectly normal nesting behaviour. The onset of nesting in normal hens occurred about 2 hr. before oviposition, and followed the lag pattern of a clutch. In birds laying soft-shelled eggs oviposition time was erratic, but nesting, although divorced from oviposition by many hours, still followed a normal lag and clutch pattern. These facts strongly suggest that ovulation time is not affected but that oviposition is induced prematurely when a foreign object is placed in the walls of the shell gland.

The way in which the thread exerts its effect is not yet known. The nature of the material is unlikely to be important, since Michel clips have a similar effect. It has been found that the foreign object must penetrate deeply into the wall of the shell gland; superficially placed threads or clips do not lead to the production of poorly shelled eggs. The contact area of the thread or clip is small relative to the mass of the shell gland and it is unlikely that irritation *per se* caused the production of soft-shelled eggs. However, it is conceivable that the effect of some local irritation could be spread by neural pathways as the uterus is well innervated (Gilbert and Lake, 1963a).

#### *The ovary in relation to oviposition*

Rothchild and Fraps (1944) showed that if the latest post-ovulatory follicle is removed, the oviposition time of the egg derived from that follicle is delayed and they suggested that a hormone was released from this structure. This effect has been confirmed (Wood-Gush and Gilbert, 1964) and it has also been shown that the results of merely ligating the stalk were the same as removal of the follicle. Cocaine injected into the stalk had a similar but less marked effect (Gilbert and Wood-Gush, 1965). This suggests that some neural mechanism is also involved and it has been found that the ovarian follicle of the hen is well innervated (Gilbert, 1965). Thus the ovary may play a more direct role in regulating the movement of the egg through the oviduct than has hitherto been thought likely.

#### *Neurohypophysial hormones and oviposition*

It is known that oxytocin and vasopressin are capable of inducing oviposition in the hen, and Tanaka and Nakajo (1962) showed that oxytocic hormones were released from the pituitary just prior to oviposition. Gilbert and Lake (1963b) also found that both oxytocin and

vasopressin became more effective in inducing premature oviposition the nearer the hen was to normal oviposition time. An aminopeptidase, capable of destroying oxytocin, was found in the blood of hens and the activity of this enzyme was found to decrease at about the time of egg-laying (Gilbert and Lake, 1964).

### Conclusion

It is generally supposed that poorly shelled eggs result mainly from a deficiency in dietary calcium, an upset in calcium metabolism or a fault in the transport of calcium to the egg in the shell gland. However, it has been shown that derangement of certain physiological mechanisms of the ovary and oviduct, which are unlikely to be related to calcium metabolism, can also lead to the production of poorly shelled eggs.

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# 3

## EFFECT OF FOWL GONADOTROPHIN FOLLOWING TREATMENT WITH A PITUITARY INHIBITOR

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### *Synopsis*

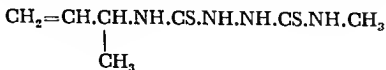
SOME of the biological properties of a non-steroid pituitary inhibitor are described, and their applicability to poultry research is discussed.

Hens maintained on a diet containing this inhibitor were injected with a crude preparation of fowl gonadotrophin, and the effects on ovary and oviduct weight and on comb size were noted. Very high dose levels were needed to stimulate development of follicles in the regressed ovary, as compared with the published requirements for ovarian maintenance in hypophysectomised hens. The range of doses examined suggested that approximately 40 mg. of the fowl extract daily for 10 days may be necessary for follicular growth. Addition of a *further one or two intravenous injections to a period of daily intramuscular injections* apparently induced ovulation, as judged by the presence of ruptured follicles in the ovary, but eggs were not laid.

Throughout the study, effectiveness of a particular hormone dose level was very variable. Suggestions are made concerning the cause of the high dose requirement, and of the variability; intended extensions of the work are outlined.

### *Introduction*

In 1961, Paget, Walpole and Richardson described some of the anti-gonadotrophin effects of I.C.I. compound 33828, a derivative of dithiocarbamoyl hydrazine, having the following formula:



These authors reported the selective inhibition of pituitary gonadotrophic activity in rats, dogs and monkeys. Normal female cycles were

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interrupted, and, in the male, accessory organs atrophied and spermatogenesis ceased. These effects were reversed when treatment ended. Later work (Brown, 1963) indicates that the compound is similarly active in several other mammals. The effective dose level varies greatly with species.

An investigation of treatment of poultry with this substance was carried out at Wye College (Sykes, 1963). He reported that feeding low dietary levels of I.C.I. compound 33828 was very effective in stopping egg production. A concentration of 0.04 per cent in the normal dry-mash ration caused egg laying to cease by the second day. Recovery took 17 to 34 days after the 10-day treatment period. This level was somewhat toxic; 0.02 per cent and 0.01 per cent were found to be equally effective; and more acceptable. Sykes's report of a preliminary investigation of exogenous gonadotrophin treatment describes very inconsistent results after a pregnant mare serum injection (Sykes, 1964).

Many studies have been reported of the effects of gonadotrophin treatment following hypophysectomy. In some of these, comparisons have been made between avian and mammalian hormone preparations. For example, Opel and Nalbandov (1961) studied the ability of gonadotrophin preparations to maintain normal ovarian function following pituitary removal. Morris and Nalbandov (1961) treated starving pullets with gonadotrophins to prevent ovarian atrophy, which usually follows removal of food. From these and other studies, it seems that chicken anterior pituitary material is superior to the purified mammalian materials tested. It is not known to what extent this can be attributed to the presence in the crude avian extract of hormones other than gonadotrophins, or to the production of immunological resistance to the mammalian preparations.

In the experiments under discussion, the I.C.I. compound was used to deplete pituitary gonadotrophins and cause ovarian regression. This was followed by treatment with fowl anterior pituitary in an attempt to restore the follicular cycle, as found in a normal active ovary, and if possible, to induce ovulation.

### *Materials and Methods*

*Fowl Pituitary.* With the cooperation of the British Egg Marketing Board, a collection of anterior pituitary glands was obtained from the Buxted Chicken Company, Ltd. The glands were removed at a broiler processing plant, from 8- to 12-week-old birds of both sexes. They were taken from fresh heads, and placed in dry acetone. Each day's collection was pooled, and stored in fresh acetone for transport to the laboratory. There, after two changes, the acetone was decanted, and the residue evaporated off at room temperature. The completely dry glands were then readily crushed to a fine pale brown powder, yielding

an average of 1.2 mg. per gland. For injection, the daily dose was suspended in 1 ml. saline.

*Experimental Animals.* The hens used in the study were adult Light Sussex hens of the Wye College flock. The selected dietary concentration of I.C.I. compound was 0.02 per cent with reference to Sykes's findings (1964). At this level, only a few birds were so badly affected as to be excluded from experiments. The remainder produced very watery droppings but were otherwise in good general condition. Food

TABLE 1

*Ovary and oviduct weights of hens fed 0.02 per cent  
I.C.I. compound 33828*

Bird No.	Period on diet (days)	Ovary wt. (g.)	Oviduct wt. (g.)
10	13	27.5	37.5
12	14	7.3	14.2
14	16	39.0	35.8
16	18	21.5	27.4
25	18	44.3	54.7
26	18	44.7	54.2
7	34	4.0	10.5
8	34	45.0	42.0
18	53	6.5	14.1
20	54	7.0	14.2
24	56	2.2	11.5
22	58	40.0	50.0

5/12 with ovary wt. less than 10 g.

4/12 with ovary wt. greater than 40 g.

consumption was rather lower than would be expected in healthy laying hens—as reported by Bayley, Clarke, Hunton and Sykes (1964)—but most of the I.C.I.-treated hens maintained body weights of around 2.5 kg., within the normal range for the flock.

*Ovarian Regression.* A preliminary experiment was carried out to investigate the period of I.C.I. compound feeding necessary to cause ovarian regression. Twelve hens were killed after periods on the diet ranging from 2 weeks to 2 months. Ovaries and oviducts were removed and weighed: these weights are shown in Table 1.

The results of this experiment indicate that the shorter periods considered were inadequate for the ovaries of most birds to reach the fully regressed state, as found in the non-laying, starved or hypophysectomised hen. The longer periods were more effective, but not consistently so; there were still some hens with quite large ovaries and oviducts. None of these birds had laid after the second day on the diet, and it was not possible to separate them by any external characters from birds with fully regressed ovaries. Higher levels of I.C.I. 33828 in the food were expected to prove toxic to a greater percentage of the birds, so it was decided not to increase the concentration.

Routine laparotomy was therefore carried out on all birds before the start of hormone injections, as a check on ovarian size. After at least 3 weeks on the diet, each bird was anaesthetised with nembutal and ether, and a small incision was made, behind and parallel to the last rib, extending dorsally as far as the ilium of the pelvis. A finger was inserted into the body cavity, to detect by touch the presence or absence of large follicles in the ovary. Although visual examination might have been preferable, this method avoided excessive retraction of viscera. Several birds were killed after laparotomy, and ovary weights recorded. These served as a check on the accuracy of the palpation method, and showed it to be satisfactory.

Four stages of ovarian development were assigned. Stage I was the fully regressed state, with no follicles greater than 0.5 cm. diameter. Stages II and III had 2 or 3, or 3 or 4 developing follicles respectively, and stage IV was an ovary with a follicular series approaching the normal state. For all experiments on stimulation of follicle growth, only birds designated Stage I or early Stage II were used. This usually meant exclusion of two or three from any group of eight birds.

Recovery from the anaesthetic was good, and a period of at least 4 days was always allowed to elapse between laparotomy and the start of hormone injections.

*Methods.* The fowl pituitary suspension was given by intramuscular injection to the pectoral muscles. The lowest dose level investigated was 5 mg. daily. Earlier work with avian gonadotrophin has usually involved freeze-dried preparations, with doses of about 5 mg. of crude extract each day. Opel (1960) reported maintenance of ovarian weight in hypophysectomised hens with 4 mg. daily. Ovulation was induced by the addition of 0.5 mg. given intravenously. In Morris and Nalbandov's work with starving pullets (1961) the dose response curve for their chicken anterior pituitary preparation suggests approximately 3 mg. daily as the best level for maintaining ovarian function. In each of these studies, exogenous gonadotrophins were supplied from the time of depletion of the endogenous hormones, and the aim was to maintain, and not to build up, the follicle series of the ovary.

In the present experiments, such small doses were without effect on the regressed ovaries. When 5 mg. was given for 4 days, and the birds killed and examined on the sixth day, no measurable effects were found. Treatment with this dose for 8 days, with examination on day 10, was little more effective.

After four injections of 10 mg., three birds from a group of eight showed some increase in ovary weight, as compared with a control group receiving no gonadotrophin, but oviduct weights were unaffected. By the tenth day, after eight injections, five of the remaining fifteen showed increased ovary and oviduct weights. The remaining ten showed little advance in the development of either organ.

In efforts to overcome this variability and find a hormone dose level

which would produce consistent ovary and oviduct enlargement, increasingly high doses were given. Intravenous injections were then added to a course of intramuscular injections, in experiments designed to stimulate growth of follicles to ovulable size, and then induce these to ovulate.

### *Experimental Results*

*Stimulation of Follicle Growth.* Table 2 shows the effects of eight daily injections of a range of doses, on the ovaries, oviducts and combs. All the birds were killed on the tenth day, the second day after the last injection. Ovaries and oviducts were removed and weighed at autopsy, and the diameters of the follicles present in the ovaries were measured,

TABLE 2

*Effect of intramuscular injection of fowl gonadotrophin on hens maintained on I.C.I. compound 33828*

Dose level mg. daily 8 days	Number of birds	Mean ovary weight (g.) ± S.E.	Mean oviduct weight (g.) ± S.E.	Mean comb change per cent.
0	8	4.9±0.91	10.9±1.11	—
10	15	30.9±9.40	32.6±3.75	+19.8
25	6	68.2±23.7	34.9±1.99	+53.3
50	6	76.1±19.8	36.2±4.60	+123.3
75	6	89.3±24.47	41.2±2.96	+117.0

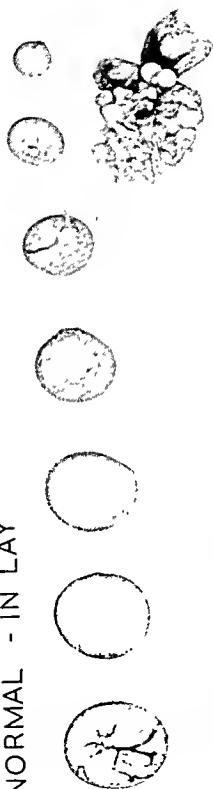
along their short axis. Records were kept of body weight changes over the period of the experiments, and also of changes in comb size, as estimated by the product of maximum comb length, and height above the eye.

As mentioned above, 10 mg. daily was inadequate. Twenty-five mg. had some stimulatory effect on all six birds of the group; ovarian weights were still very variable, but the effect on the oviduct was more consistent. With both 50 and 75 mg., ovarian reaction was clearly divided. Four hens out of six in each case had greatly enlarged ovaries, and two of the six lacked follicular growth almost completely, beyond follicle diameters of 0.5 to 1.0 cm. This could not be related to general poor condition of individual birds. Greater consistency of oviduct weight was again found. Differences between experimental and control values for mean weights of ovary and oviduct were tested for significance by Student's *t* test. At the 1 per cent level of probability, all the oviduct weight means were significantly greater in experimental groups than in the controls, but only the 25, 50 and 75 mg. treatments produced significantly heavier ovaries. The 25 mg. difference only just achieved significance. The greatest mean comb size increase, expressed as a percentage of the comb size at the start of the experiment, was recorded with 50 mg. daily injection.

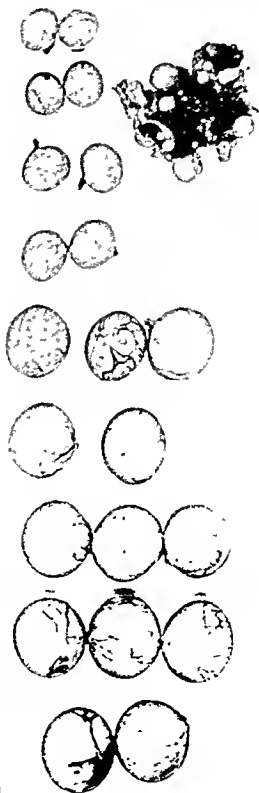
The qualitative effects on the ovaries are summarised in Table 3.



# NORMAL - IN LAY



# OVERSTIMULATED



FOILICLES FROM OVARIES OF TREATED AND NORMAL HENS

The ovary of a normal laying hen is shown, with its follicles detached. Below this are the overstimulated ovary and follicles from an experimental bird receiving 75 mgm. fowl pituitary powder daily.

In the 10 mg. group, although almost half the ovaries were apparently unstimulated, one hen ovulated and produced a normal egg on day 9. The ovary of this hen was classified as overstimulated at autopsy. A normal egg was also produced during the 25 mg. experiment, again from an ovary which appeared overstimulated, and in the vary of another hen in this group a recently ruptured follicle was found, but no egg was recorded, or found in the oviduct. A soft-shelled egg may have been lost through the cage floor, or the yolk may have been resorbed from the body cavity.

TABLE 3

*Summary of effect of fowl gonadotrophin injections on I.C.I. 33828-regressed ovaries*

Treatment level mg. daily— 8 days	Proportion of ovaries			Ovulations
	Overstimulated	Apparently normal	Lacking follicle growth	
10	2/15	6/15	7/15	1 normal egg laid
25	2/6	2/6	2/6	1 normal egg laid 1 recently ruptured follicle in ovary
50	2/6	2/6	2/6	0
75	4/6	1/6	1/6	1 soft-shelled egg laid 1 normal egg in shell gland +1 ruptured follicle in ovary

Although no signs of ovulation were found in the 50 mg. experiment, a similar result was obtained with the 75 mg. dose level. This was the highest level given. Here, one hen produced a soft-shelled egg on the eighth day, and another was found to have a complete egg, with fully formed shell, in the shell gland, and two recently ruptured follicles in an apparently normal ovary. Thus, one yolk appeared to have been lost, by resorption, or as a soft-shelled egg which was not recorded.

In these last two experiments, the ovaries which did react by follicular development tended to be greatly enlarged, and weighed more than would be expected of a normal ovary from a laying hen. This was due to general overstimulation of all follicle sizes. A graded series of sizes was found, but with two or three representatives of each size, instead of one or two, as is normal (see Plate 1).

*Induction of Ovulation.* In the overstimulated ovaries, follicles often approached the size at which a normal follicle would ovulate. Two intravenous injections of the fowl pituitary were therefore added at the end of a course of intramuscular injections, in an attempt to cause ovulation of the largest such follicles. The injection pattern followed was then eight intramuscular, followed by two intravenous; the hens were killed on the twelfth day.

The dose level of 50 mg. was selected to produce follicular growth, without excessive overstimulation. Addition of two intravenous injections of 10 mg. was ineffective, so in a second experiment, intravenous doses of 50 mg. were given. Ovarian development was recorded in all six birds of the group; mean ovary weight was  $91.25 \pm 8.39$  g., and mean oviduct weight,  $40.95 \pm 3.65$  g. Both values were significantly greater than control values at the 1 per cent probability level. Although none of the six laid, four of them were found to have recently ruptured follicles in the ovary. No oviducal eggs were found. In five of these six, atresia had set in, in the larger follicles, but was not far advanced, and had not reached the smaller members of the follicular series, as judged macroscopically. As in the experiments with intramuscular injections alone, there was more than one representative of each size class.

### *Discussion and Conclusions*

These experiments indicate that intramuscular injections of a crude fowl pituitary preparation can induce follicular growth in a regressed ovary. Over a 10-day experimental period, a dose level of 25 mg. for 8 days was only partially effective. Fifty mg. resulted in some overstimulation, and may have been slightly higher than necessary; 75 mg. produced marked overstimulation. In these experiments, some individual hens failed to respond by follicular development. From these results, the possibility emerges that a dose level of about 40-45 mg. daily might be expected to give good results. The appearance of recently ruptured follicles after the addition of intravenous injections to a course of intramuscular treatment suggests the induction of ovulation, despite the absence of oviposition in all hens receiving such treatment. As ovulation and oviposition were recorded during experiments with intramuscular injections alone, the possibility cannot be ignored that some at least of these ruptured follicles may have been evidence of spontaneous ovulation, unrelated to the intravenous injections.

The dose levels used in this series of experiments appear extremely high when compared with those in other published data on fowl pituitary treatment of hypophysectomised hens. A number of suggestions can be made in an attempt to explain this. Firstly, such an effect as this would be seen if the extract used were of low potency. Secondly, a partial inhibition by I.C.I. 33828 of exogenous gonadotrophin might be suggested. A third possibility is that the hormone requirement for follicular growth from the fully regressed state, in a 10-day period, is much higher than the requirement for maintenance of an existing follicle series. These possibilities may be considered, and future experiments are planned in the hope of enabling one or more to be discarded. On the evidence available for human and other pituitary glands, it is not likely that acetone-drying, as opposed to freeze-drying,

caused a great loss of potency. Trial assays of the fowl material have been carried out by Dr Butt at the Birmingham Department of Clinical Endocrinology. The assay techniques used involved the responses of ovaries and uteri of immature female mice to the test substance, and preliminary results do not indicate low potency of the preparation in follicle-stimulating hormone or in total gonadotrophin.

The possibility that the I.C.I. compound counteracts exogenous gonadotrophins in fowl has not been investigated. Paget *et al.* (1961) originally reported that it did not counteract mammalian hormone activity in immature rats. However, Brown (1963) reported some effect on the slope of the dose response lines when large doses of I.C.I. 33828 were given together with gonadotrophins in assay experiments, but suggested that toxicity effects might have been responsible for this.

In these experiments oviduct weights were much more consistent than ovary weights, and might be greatly increased as compared with controls even when the ovaries appeared to be unaffected. The oviducts were not examined at laparotomy, so there was no direct evidence that oviduct weights were low initially. However, oviducts in controls with regressed ovaries were consistently small. It would seem that although follicle growth as judged by yolk deposition may have been absent, such high hormone levels did have an effect on the endocrine activity of the ovary, to produce stimulation of oviduct and comb growth. A histological study of the ovaries is to be carried out. A food intake study of fowl treated with I.C.I. 33828 together with gonadotrophin might determine whether the food intake of these birds is adequate for yolk synthesis and deposition.

Another point is the absence of ovulation in some birds with large follicles present. This might be related to an overstimulation effect, or to an inadequacy of ovulation inducing hormone at the appropriate time. Judgment of this must be deferred until more information is available about potency and relative hormone content of the pituitary preparation. The experimental approach to the problem has been suspended for the present, to permit assay work and histological examination of tissues to proceed.

#### *Acknowledgements*

Thanks are due to the British Egg Marketing Board and to the Buxted Chicken Company Ltd for the collection of the fowl pituitaries, and to I.C.I. Pharmaceuticls Division for supplies of compound 33828.

This work was carried out at Wye College under the supervision of Dr A. H. Sykes, during tenure of a British Egg Marketing Board Studentship.

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## OESTROGENS IN PLASMA FROM THE DOMESTIC FOWL

J. E. O'GRADY

*Twyford Laboratories Ltd., Twyford Abbey Road, London, N.W.10**Synopsis*

A DOUBLE isotope derivative technique is described for the identification of plasma oestrone, oestradiol and oestriol. Plasma from the laying domestic fowl was hydrolysed in acid, extracted with chloroform and the phenolic steroids isolated from this extract. The oestrogens in the phenolic steroid extract were converted to their 3-methyl ethers with T-dimethyl sulphate, mixed with authentic C<sup>14</sup> methyl oestrogens and purified by six chromatographic steps. The isolated methyl oestrogens displayed a constant C<sup>14</sup>/T ratio in the last three chromatographic transfers showing that radiochemical homogeneity had been reached. Using this technique both oestrone and oestradiol were shown to be present in plasma from laying birds but oestriol could not be detected.

*Introduction*

Although there exists an extensive literature concerned with the effects of oestrogenic substances on the domestic fowl, surprisingly little is known about the nature of the oestrogens circulating naturally in the laying bird.

Thus although the ovaries of the laying domestic fowl were shown to contain oestrone, oestradiol and oestriol (Layne, Common, Maw and Fraps, 1958), and oestrone and oestradiol have been isolated from extracts of laying hen urine (Ainsworth and Common, 1962; Macrae, Saharia and Common, 1959), the precise nature of the plasma oestrogens is unknown. Layne *et al.* (1958) subjected extracts of whole blood (650 ml.) to paper chromatography to separate and isolate the oestrogens and used ultraviolet absorption spectrometry to identify the isolated materials. They found that a compound identical with or very similar to oestrone was present in the conjugated steroid fraction of the blood but were unable to detect oestradiol and oestriol in any of the blood steroid fractions.

The technique of double isotope derivative analysis has the advantages of greater sensitivity and specificity over more conventional

chemical analytical procedures. Consequently this technique has been used in an attempt to identify the plasma oestrogens of the domestic fowl. This study was undertaken as part of an investigation into the factors involved in ovulation and yolk production in the laying bird.

### *Materials and Methods*

*Reagents.* All the reagents employed were of Analar grade. Alumina (Savory and Moore Ltd., London) of mesh size 100/150 was deactivated with 5-10 per cent of water and adjusted for chromatography by the procedures described by Roy and Brown (1960). Silica gel for thin layer chromatography was Kieselgel G (A. G. Merck, Darmstadt).

Inert methyl oestrogens (Sigma Chemical Co., London) were re-purified by alumina chromatography (Brown, 1955).

T-dimethyl sulphate (Radiochemical Centre, Amersham) was of specific activity 100 mc./mM. 6-C<sup>14</sup> oestrone (Radiochemical Centre Amersham) and C<sup>14</sup> oestradiol are of specific activity 10 mc./mM. C<sup>14</sup> methyl oestrogens were prepared by methylating the appropriate free oestrogen with inert dimethyl sulphate and purification of the product by alumina chromatography using the procedure devised by Brown (1955).

*General Technique.* All solvents were evaporated at 40°C. in a rotary vacuum evaporator. Alumina chromatography was performed in chromatography tubes 11 mm. × 5 mm. diameter sealed on to a reservoir of approximately 25 ml. capacity. The alumina was retained in the tube with porous polystyrene discs. The apparatus used for thin layer chromatography was manufactured by Desaga, Heidelberg, Germany and retailed by Camlab Glass Ltd.

*Plasma.* The birds used for the supply of plasma were a White Leghorn Cross (CCA × CRM) and were approximately 30 weeks of age. Samples of blood (25 ml.) were taken from each bird by bleeding from the jugular vein and collected in beakers containing 100 mg. sodium citrate crystals. After centrifugation the plasma from six birds was pooled and 50 ml. portions used in each study.

*Isolation and Identification of Oestrogens.* Plasma (50 ml.) was hydrolysed by heating under reflux with a mixture of distilled water (250 ml.) and 10 ml. conc. hydrochloric acid (11 N) for 1 hr. The phenolic steroids were extracted and isolated from the hydrolysate by the following procedure which is a modification of a technique for the purification of oestrogens from plasma devised by Svendsen (1960). Fig. 1 is a flow sheet of the modified procedure. After cooling, the plasma hydrolysate was extracted with three volumes (75 ml.) of chloroform. The final emulsion was centrifuged at 1,020 g for 10 min. and the lower chloroform layer added to the previous extracts. Water was removed from the extract with 10 g. anhydrous sodium sulphate and the extract was then evaporated to dryness. The dried lipid material was

transferred to a test tube with 10 ml. chloroform/methanol (1:1 v/v) and the solvent again evaporated. Excess lipid was removed by partition between ethanol-water (1:1 v/v) (6 ml.) and *n*-pentane (6 ml.). The lipid which passed into the pentane phase was discarded. The pentane partition was twice repeated and the ethanolic phase was then dried. The partially purified steroids were taken up in 3 ml. of carbon

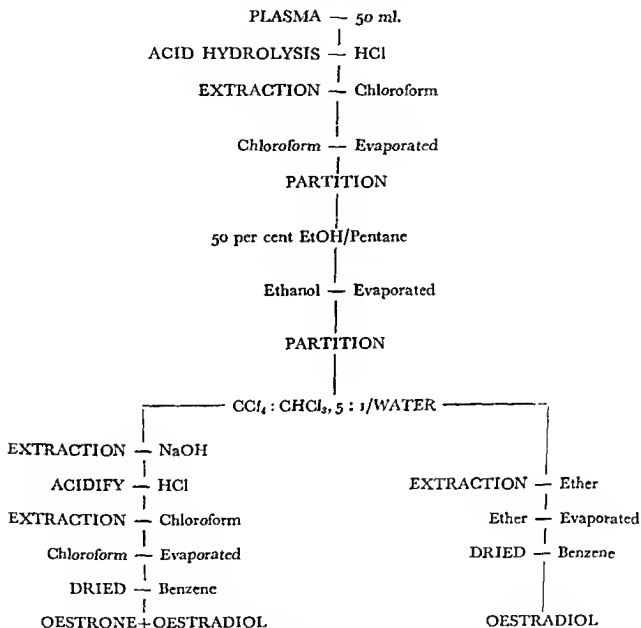


FIG. 1. Flow sheet of the initial procedures employed in the isolation and identification of plasma oestrogens.

tetrachloride/chloroform (5:1 v/v) and the solution extracted with three volumes (15 ml.) of distilled water. This latter partition retained oestrone and oestradiol in the organic phase whereas oestriol was extracted into the aqueous phase. The organic phase was extracted with three volumes of 1N sodium hydroxide (1.5 ml.) when the phenolic steroids passed into the aqueous alkaline solution. After the alkaline extract had been acidified with conc. hydrochloric acid (1 ml.) it was extracted with three volumes of chloroform (1.5 ml.) when the steroids passed back into the organic phase. The final chloroform solution was washed first with 1.5 ml. disodium hydrogen phosphate (0.1 M, pH 9),



followed by 1.5 ml. distilled water and finally evaporated to dryness. The final residue containing the phenolic steroids was further dried by repeated addition and evaporation of 1 ml. portions of anhydrous benzene. The aqueous solution containing oestriol was extracted with three volumes (2 ml.) of diethyl ether. The ether extract containing oestriol after evaporation of the organic solvent was dried with benzene as described above.

*Methylation.* The purified oestrogens obtained as above were incubated in lightly stoppered tubes at 40°C. for 48 hr. with 1  $\mu$ mole of T-dimethyl sulphate dissolved in 0.1 ml. anhydrous benzene, and 0.1 ml. of 1 per cent (w/v) suspension of anhydrous potassium carbonate in acetone. After incubation the solvents were evaporated and the residual material shaken with 0.5 ml. 1 N sodium hydroxide solution. The tritiated methyl oestrogens were extracted from the aqueous solution with three volumes (0.5 ml.) of *n*-hexane. Any "free" oestrogen remained in the alkaline solution. The hexane extract was finally washed with three volumes (0.5 ml.) of water.

*Final Purification.* To the extract of the T-methyl oestrogens, diluted to 5 ml. with *n*-hexane saturated with water, 10  $\mu$ g. of each inert carrier methyl oestrogen and 1 m $\mu$ c. of each C<sup>14</sup> methyl oestrogen were added and the mixture was subjected to six chromatographic steps.

Fig. 2 is a flow sheet representing the order of chromatography. Chromatography on alumina was performed as described by Brown (1955). The solvents used in elution of the columns were collected in fractions of 1.0 ml. and 0.1 ml. portions of these were taken for the determination of radioactivity. The procedures for thin layer chromatography were those of Lisboa and Diezfelusy (1962). The thin-layer chromatograms were run for 10 cm., removed from the chromatography tank and dried in air. Standard oestrogens were run on each chromatogram to aid in locating the unknown oestrogen spots. The standards were located by taking eight 0.5 cm. fractions in the area normally occupied by the R<sub>f</sub> of the oestrogen in question. The silica gel from each fraction was eluted twice with 1 ml. of ethanol. Portions of each fraction (0.2 ml.) were taken for liquid scintillation counting of its radioactivity. Having located the standard oestrogens the corresponding area of silica gel on the unknown chromatograms were removed, eluted and their radioactivity counted by the procedures employed in the location of the standards. The fractions containing the individual unknown methyl oestrogens after each chromatographic step were dried in a rotary evaporator, redissolved in ethanol (50  $\mu$ l.) and applied to the origin of the next chromatogram. A modified procedure was employed in the elution of the final thin layer chromatogram. The position of the methyl oestrogen was located by running a C<sup>14</sup> methyl oestrogen standard next to the unknown material on the same chromatographic plate and eluting this standard by the technique described above. This method approximately located the active spot

which was divided in an origin, a centre and a front region. These three regions were separately scraped from the plate and eluted with ethanol as previously described.

*Determination of Radioactivity.* The radioactivity of each fraction was determined by a liquid scintillation technique. The portion of each chromatographic fractions taken for scintillation counting was pipetted

# OESTRONE+OESTRADIOL AND OESTRIOL EXTRACTS

## METHYLATION — TRITIATION

### T-DIMETHYL SULPHATE

#### PARTITION

HEXANE/NaOH 1N ————— DISCARD

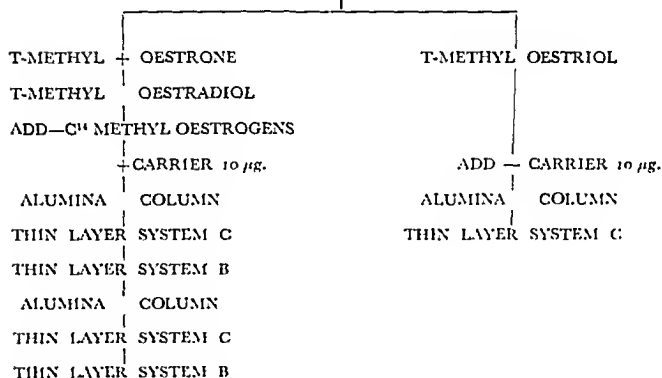


FIG. 2. Flow sheet of the methylation and chromatography employed in the isolation and identification of plasma oestrogens.

into a silica counting vial. Scintillation liquid (NE 220, Nuclear Enterprises, Edinburgh) (4 ml.) was added to the vial and after swirling to ensure complete homogeneity, the samples were counted in an automatic double channel scintillation counter (Nuclear Enterprises, Edinburgh), to an accuracy of 1.99 per cent for tritium and 1.2 per cent for  $C^{14}$ .

chromatography indicating that the oestrogen had become radiochemically pure. The  $T/C^{14}$  methyl oestradiol also attained chromatographic purity after the second alumina chromatography, the isotopic ratio for this steroid remaining constant throughout the subsequent chromatographic steps. The homogeneity of the  $T/C^{14}$

TABLE 1  
*Isotopic ratios of  $T/C^{14}$  methyl oestrogens during chromatographic purification*

Chromatographic system	$T/C^{14}$ methyl oestrone			$T/C^{14}$ methyl oestradiol		
	$C^{14}$ cpm.	T cpm.	$T/C^{14}$ ratio	$C^{14}$ cpm.	T cpm.	$T/C^{14}$ ratio
1. Alumina column	2,164	14,716	6.80	2,063	25,998	12.6
2. TLC—C	1,903	6,340	3.23	1,641	10,666	6.5
3. TLC—B	1,741	3,483	2.00	1,564	7,197	4.6
4. Alumina column	1,262	2,323	1.84	843	1,011	1.20
5. TLC—C	841	1,582	1.88	712	861	1.21
6. TLC—A	213	394	1.84	566	662	1.17

methyl oestrogens was confirmed by the constancy of the  $T/C^{14}$  ratio of the three fractions obtained during the final thin layer chromatography of both these steroids (Table 2).

After the initial alumina chromatography no tritium was observed in the chromatographic position normally occupied by methyl oestriol. This result indicates that oestriol is not present in the plasma of the laying bird. However, as no  $C^{11}$  methyl oestriol standard was available,

TABLE 2  
*Isotopic ratios across the final "spot" of  $T/C^{14}$  methyl oestrogen obtained during the last stage of chromatographic purification.*

	$T/C^{14}$ methyl oestrone			$T/C^{14}$ methyl oestradiol		
	$C^{14}$ cpm.	T cpm.	$T/C^{14}$ ratio	$C^{14}$ cpm.	T cpm.	$T/C^{14}$ ratio
Origin	46	85	1.84	141	166	1.17
Centre	98	181	1.85	367	426	1.16
Front	69	128	1.86	58	70	1.20

the recovery of this steroid during the extraction, methylation and purification processes was unknown and may have been too low to allow detection.

These results provide evidence that both oestrone and oestradiol circulate naturally in the plasma of the laying domestic fowl. Previous work of Layne *et al.* (1958) suggested that oestrone may be present in the plasma of the laying domestic fowl in a conjugated form. This investigation supports the contention that oestrone is present in the plasma, but since hydrolysis was used before extracting with chloroform it is not possible to tell whether the steroids occur naturally in the free or bound condition.

It is hoped that after further development, it will be possible to employ the techniques described as the basis of a quantitative method of analysis for peripheral plasma oestrogens.

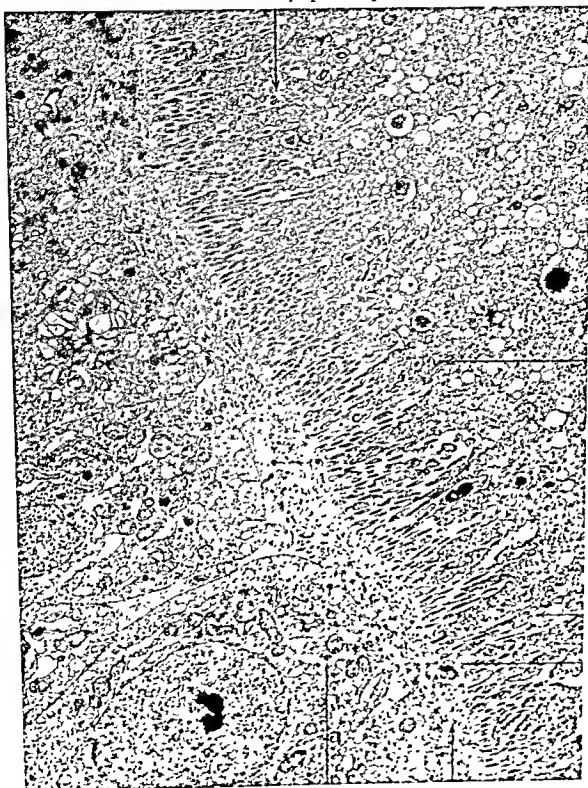
### *Acknowledgements*

It is a pleasure to thank Cyril Thornber Ltd, Mytholmroyd, Halifax, Yorkshire, for a supply of birds.

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Cytoplasmic process



Granulosa  
process

} Zona  
radiata

Granulosa  
cell

Perivitelline  
substance

Section of 7 mm follicle showing granulosa cells, perivitelline substance and zona radiata

7, 270

# SOME OBSERVATIONS ON THE FINE STRUCTURE AND HISTOCHEMISTRY OF THE OVARIAN FOLLICLE OF THE FOWL

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## *Synopsis*

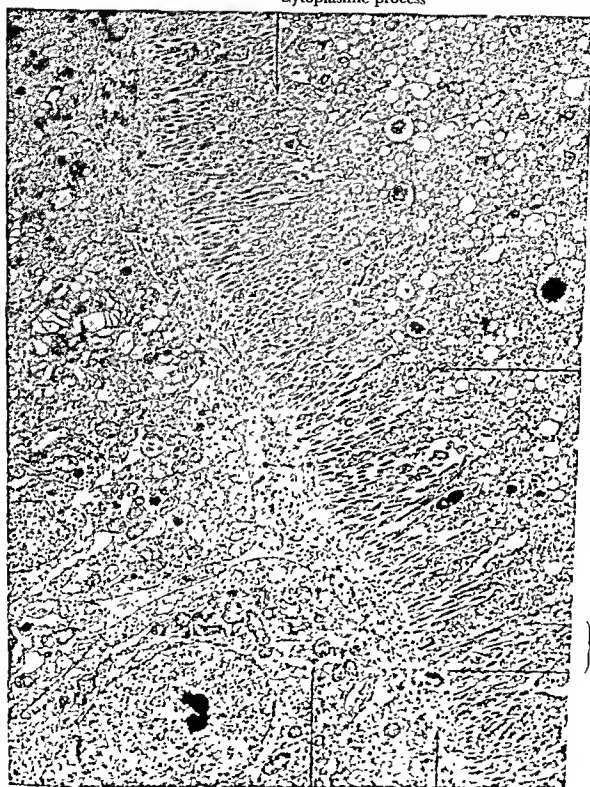
IN AN EARLY growing hen's follicle the ovum and the granulosa cells become separated by a perivitelline layer of homogeneous substance and the zona radiata consists of primary and secondary processes of ovum cytoplasm plus cytoplasmic processes of the granulosa cells with modified plasma membranes. At a later stage the perivitelline substance fills wide intercellular spaces between the granulosa cells and extends into the zona radiata between the cytoplasmic processes of the ovum. The structural characteristics of the post-ovulatory granulosa cells are strikingly different from those of the pre-ovulatory granulosa cells.

3 $\beta$ -, 11 $\beta$ -, 17 $\beta$ - and 20 $\beta$ -hydroxysteroid dehydrogenases have been demonstrated histochemically in the granulosa and theca interna, and there appears to be no striking change in the distribution of these enzymes between the pre- and post-ovulatory follicles. The existence of these pathways points to progesterone, and possibly oestrogen, synthesis in this tissue.

## *Introduction*

There are two problems of special interest in connection with the development of the follicle in the hen's ovary. First, the mechanism of the rapid accumulation of yolk within the cytoplasm of the ovum whereby the follicle is increased in size from 2 mm. to 40 mm. in diameter in a few days before ovulation. Second, the question of the secretion of ovarian hormones before and after ovulation. The electron microscope has made it possible to observe the fine structure of the growing follicle for evidence of adaptations to deal with the special physiology of the hen's follicle and new histochemical techniques can now demonstrate the presence of the specific enzymes concerned in relevant pathways of steroid metabolism to indicate which follicular cells are equipped to produce oestrogen and progesterone.

Cytoplasmic process



Granulosa  
process

Zona  
radiata

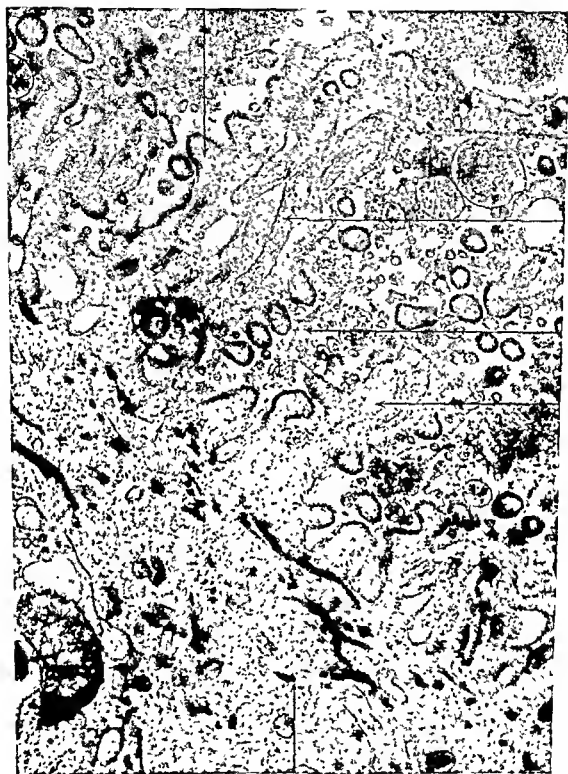
Granulosa  
cell

Perivitelline  
substance

Section of 7 mm follicle showing granulosa cells, perivitelline substance and zona radiata

7.4.70

Pinocytosis



Granulosa  
process

Cytoplasmic  
process

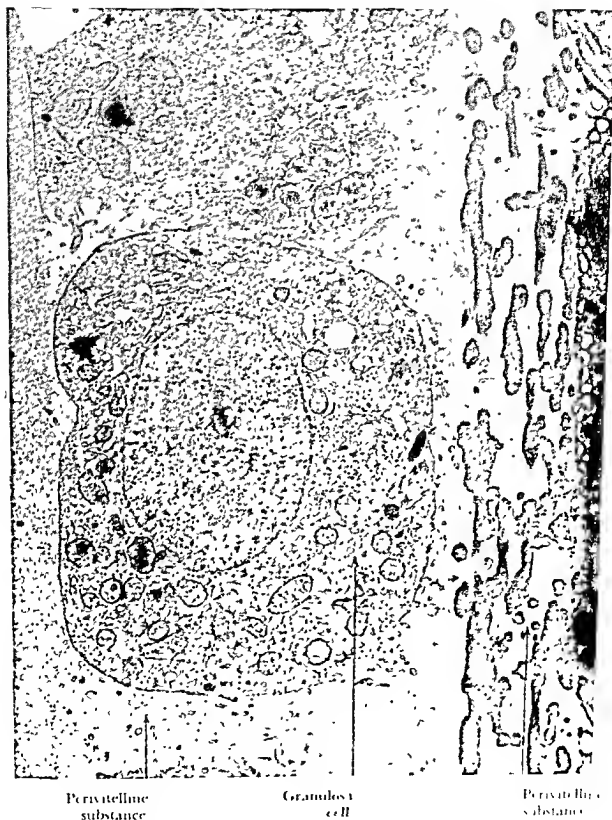
Perivitelline  
substance

Perivitelline  
substance

Section of 15 mm. follicle showing zona radiata with processes of perivitelline substance. Specialisation of the plasma membrane of the ovum may indicate a process of pinocytosis.

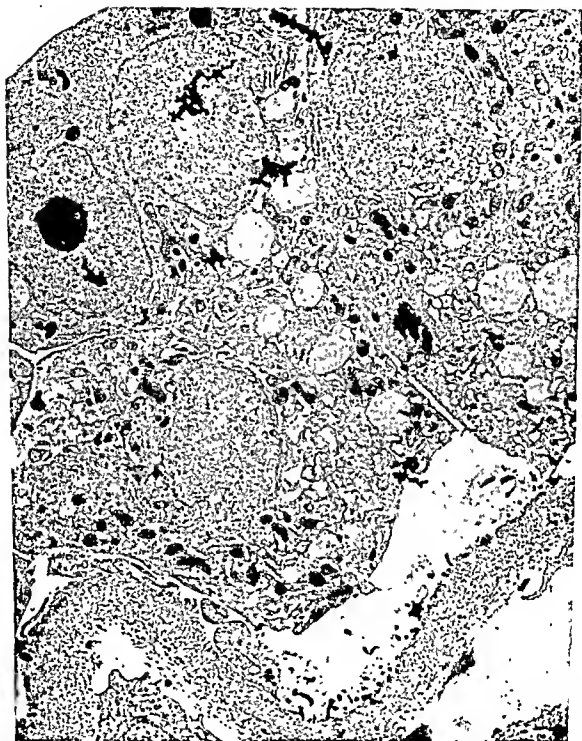
× 16,800





Section of 35 mm follicle showing perivitelline substance with rods of dense material and granulosa cells

W. J. P.



Section through wall of 48 hour post-ovulatory follicle showing granulosa cells with partially detached basement membrane.

$\times 8,000$

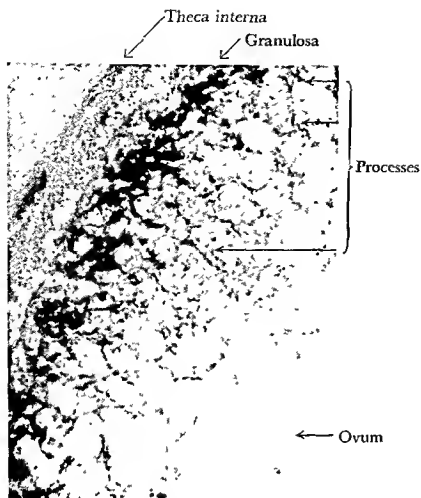


PLATE 6. Follicle, 24 hours, pre-ovulatory. Intense  $3\beta$ -hydroxysteroid dehydrogenase activity is seen in the theca interna and granulosa: note particularly the reactive processes of granulosa cells extending into the ovum. Pregnenolone.

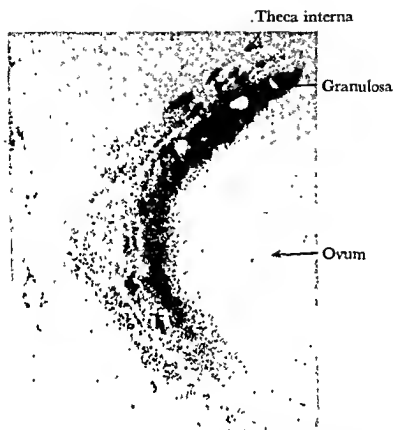
$\times 105$



PLATE 7

Follicle, immediately post-ovulatory. Intense  $3\beta$ -hydroxysteroid dehydrogenase activity is seen in the theca interna and granulosa: note particularly the reactive processes of granulosa cells extending into the ovum. Pregnenolone sulfolate.

$\times 105$



Follicle, early.  $11\beta$ -hydroxysteroid dehydrogenase activity is visible in the theca interna and granulosa.  $11\beta$ -hydroxyprogesterone.  $\times 65$

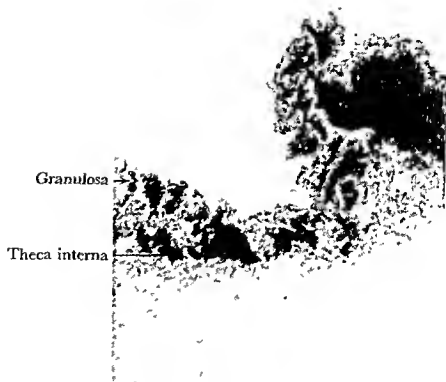


PLATE 9

Follicle, 72 hour post-ovulatory.  $11\beta$ -hydroxysteroid dehydrogenase activity in the theca interna and in a few adhering granulosa cells.  $11\beta$ -hydroxyprogesterone.  $\times 105$

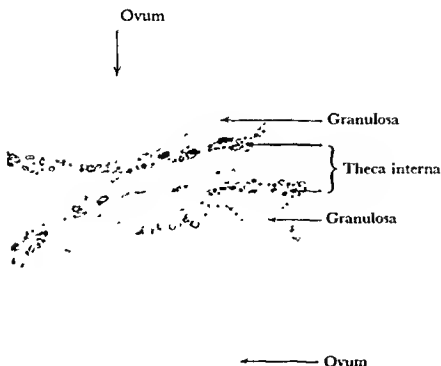


PLATE 10

Two adjacent early follicles.  $20\beta$ -hydroxysteroid dehydrogenase activity in the theca interna.  $20\beta$ -hydroxyprogesterone.  $\times 65$

*Material and Methods*

Follicles were obtained from sexually mature laying Thornber 505 hybrid hens of approximately 18 months of age. These hens ovulated once per day and it is possible, therefore, by measuring and counting the actively growing ova to determine the approximate pre-ovulatory age of individual follicles. The follicles studied measured 2 mm. (i.e. a follicle during the period of slow growth), 7 mm., 15 mm. (5 days before ovulation), and 35 mm. (mature follicle).

The follicles were excised from anaesthetised hens and fixed in either 1 per cent buffered isotonic osmic acid (Zetterquist, 1956), or 5 per cent buffered glutaraldehyde, followed by osmic acid. In the larger follicles, because of a tendency for the granulosa layer to become detached from the underlying theca, fixative was first injected and thereafter large pieces were placed in fixative. Since the wall of the larger follicles is very thin, this allowed adequate penetration of fixative and permitted proper orientation prior to embedding.

Tissues were dehydrated through ascending grades of methanol and embedded in araldite. Sections were cut on either an LKB or Porter-Blum microtome and picked up on an uncoated copper grid. They were stained in methanolic solutions of either lead acetate or uranyl acetate for from 20 min. to 2 hr., rinsed in methanol and allowed to dry at room temperature. The sections were screened in a Philips E.M. 200 at 60 kV.

For evidence of steroid production by the components of the follicles, histochemical methods, now established for the demonstration of the relevant enzymes, namely  $3\beta$ -,  $11\beta$ -,  $17\beta$ - and  $20\beta$ -hydroxysteroid dehydrogenases, were used. Seven stages in the life cycle of the follicle, corresponding to the quiescent, the growing and post-ovulatory phases, were tested.

Follicles were excised, snap frozen on carbon dioxide and sectioned at  $15\ \mu$  in a cryostat maintained at  $-20^\circ\text{C}$ . The sections were attached to slides by momentary thawing and before incubation were brought to room temperature and dried in air. A positive result shows as pink monoformazan or blue diformazan. Sections from each follicle were examined to demonstrate the presence of the enzymes listed below using the appropriate substrate; control sections were incubated at the same time with propylene glycol but no steroid substrates and incubations lasted 30 min. Positive controls were not encountered in this tissue.

(1)  $3\beta$ -Hydroxysteroid dehydrogenase (Wattenberg, 1958) incubated with the following steroids in solution in propylene glycol:

- (a)  $3\beta$ -Hydroxypregn-5-en-20-one (Pregnenolone)
- (b)  $3\beta$ ,  $17\alpha$ -Dihydroxypregn-5-en-20-one ( $17\alpha$ -hydroxypregnenolone)
- (c)  $3\beta$ -Hydroxyandrost-5-en-17-one (DHA)

- (d) Androst-5-ene-3 $\beta$ , 17 $\beta$ -diol (Androstenediol)
- (e) 3 $\beta$ -Sulphoxy pregn-5-en-20-one (Pregnenolone sulphate)
- (f) 3 $\beta$ -Sulphoxy-17 $\alpha$ -hydroxy pregn-5-en-20-one (17 $\alpha$ -hydroxy-pregnenolone sulphate)
- (g) 3 $\beta$ -Sulphoxy androst-5-en-17-one (DHA sulphate) cholesterol
- (h) 3 $\beta$ -Acetoxy cholesterol (Cholesterol acetate)
- (i) 3 $\beta$ -Acetoxypregn-5-en-20-one (Pregnenolone acetate)
- (j) 3 $\beta$ -Acetoxy androst-5-en-17-one (DHA acetate)

(2) 11 $\beta$ -Hydroxysteroid dehydrogenase (Baillie, Ferguson, Calman and Hart, 1965). 11 $\beta$ -Hydroxyprogesterone and 11 $\beta$ -hydroxy-androstenedione dissolved in propylene glycol were employed as substrates.

(3) 17 $\beta$ -Hydroxysteroid dehydrogenase (Pearson and Grose, 1959). Oestradiol and testosterone were used as substrates dissolved in propylene glycol.

(4) 20 $\beta$ -Hydroxysteroid dehydrogenase (Baillie, Calman, Ferguson and Hart, 1965). The substrate used was 20 $\beta$ -hydroxyprogesterone.

Haematoxylin and eosin preparations of each follicle were routinely examined to confirm the naked eye estimate of its age.

## Results

### *Electron Microscopic Observations*

It has been shown (Wyburn, Aitken and Johnston, 1965) that in a follicle at an early stage of growth (Plate 2), the ovum and granulosa cells have become separated by a layer of homogeneous substance, the perivitelline layer, probably secreted by the granulosa cells. At the junctional zone the cytoplasm of the ovum forms primary processes which in turn break up into secondary processes containing numerous membrane bounded vesicles. This is the zona radiata. Cytoplasmic extensions from the granulosa cells penetrate deeply into the zona radiata but remain separated from the ovum cytoplasm by specially modified plasma membranes. At a later stage (Plate 3) the perivitelline substance has increased and occupies the wide intercellular spaces now separating the granulosa cells and extends into the zona radiata between the cytoplasmic processes of the ovum.

In an immediately pre-ovulatory follicle (Plate 4) the granulosa cells remain widely separated by intercellular spaces filled with the perivitelline substance. There is now no zona radiata which has become obliterated by the extension of the cytoplasm of the ovum to the wall of the follicle. In the pre-ovulatory follicle there is a single layer of granulosa cells separated from the theca interna by a thick basement membrane. In the theca interna there are the so-called luteal cells characterised by their content of large membrane-bounded granules.

The granulosa cells have abundant Golgi substance, numerous

active looking mitochondria and the cytoplasm is packed with both granular and smooth endoplasmic reticulum. There are cytoplasmic processes extending from the apical surface of the granulosa cells into the perivitelline substance. Granulosa cells are still found in the wall of the follicle 72 hr. after ovulation but they now look quite different from those of the pre-ovulatory follicle and resemble rather the lutein cells of the mammalian corpus luteum. Their cytoplasm is filled by a network of spaces and large dense membrane-bounded lipid granules (Plate 5).

### *Histochemical Results*

#### (1) $3\beta$ -Hydroxysteroid dehydrogenase

##### *The free steroids*

After incubation with pregnenolone,  $3\beta$ -hydroxysteroid dehydrogenase activity is readily demonstrable in all the follicular stages studied. The granulosa cells of the early follicle contain minute precipitates of blue diformazan and their cytoplasmic lipids colour pink due to pooled monoformazan. Processes from the free margin granulosa cells (Plate 6) extend quite long distances into the cytoplasm of the ovum and are rendered conspicuous by the abundance of their contained diformazan. Mono- and diformazan deposits are also a prominent feature of the theca interna cells. This reactivity does not alter as ovulation approaches. Intense reactivity in the theca interna is visible in the immediately post-ovulatory follicle and this activity is markedly reduced in the ensuing 72 hr. The general ovarian stroma contained a few weakly reactive cells.

With  $17\alpha$ -hydroxypregnenolone as substrate a very poor histochemical reaction was observed in the granulosa cells and theca interna at all stages.

The histochemical appearance following incubation with DHA and androstenediol closely resembles that obtained with pregnenolone, intense  $3\beta$ -hydroxysteroid dehydrogenase activity being visible in the cells of the granulosa and theca interna, although the colour obtained with androstenediol may in part come from the  $17\beta$ -hydroxyl group.

##### *The steroid sulphates*

Using pregnenolone sulphate as substrate (Plate 7)  $3\beta$ -hydroxysteroid dehydrogenase was demonstrable in the granulosa cells and their intraovular processes and in the theca interna. The reaction developed more quickly than with the free steroid.

The reaction obtained with  $17\alpha$ -hydroxypregnenolone sulphate as a histochemical substrate developed as rapidly and was as intensely positive as that obtained with pregnenolone sulphate and very much more heavily positive than the reactivity with  $17\alpha$ -hydroxypregnenolone. No age changes were seen with either of these sulphates.



Using DHA sulphate a trace of monoformazan was observed in some granulosa cells. This steroid, however, was not used to any extent by the granulosa or theca interna cells.

#### *Steroid acetates*

Cholesterol acetate was not used histochemically in any of the follicles except the very early follicle. In the extremely early follicle minute diformazan deposits were seen in the granulosa cells and a few diformazan crystals were seen in the theca interna. All the later follicles are completely inactive with respect of this substrate.

Using pregnenolone acetate and DHA acetate,  $3\beta$ -hydroxysteroid dehydrogenase activity is visible in the granulosa and theca interna of all follicles studied. Mono- and diformazan deposits are readily seen after brief incubation and the reaction is uniformly strong up to and including the immediately post-ovulatory follicle. Reactivity seemed somewhat reduced in the two later post-ovulatory follicles.

#### (2) $11\beta$ -Hydroxysteroid dehydrogenase (Plate 8)

Both  $11\beta$ -hydroxyprogesterone and  $11\beta$ -hydroxyandrostenedione were used by the granulosa and theca interna of all the follicles studied but monoformazan mainly was deposited and the reaction was considerably less well developed than the  $3\beta$ -hydroxysteroid dehydrogenase activity. No changes were noted with age in the follicle.

#### (3) $17\beta$ -Hydroxysteroid dehydrogenase (Plate 9)

Incubation with oestradiol or testosterone resulted in mono- and diformazan being deposited in the granulosa and theca interna of all follicles studied. As with the  $11\beta$ -hydroxysteroid dehydrogenase the reaction was much weaker than the  $3\beta$ -hydroxysteroid dehydrogenase and did not change appreciably with age.

#### (4) $20\beta$ -Hydroxysteroid dehydrogenase (Plate 10)

This enzyme was very poorly reactive in the theca interna and granulosa of all follicles studied. Monoformazan only was deposited.

### *Discussion*

The electron microscope has revealed that the structure of the growing ovum is admirably adapted for the absorption of substances necessary for the rapid accumulation of yolk at this time. On the basis of such observations it has been suggested (Wyburn, Aitken and Johnston, 1965) that the perivitelline substance is precursor yolk elements and is absorbed into the cytoplasm of the ovum possibly by pinocytosis of the plasma membrane of the cytoplasmic processes of the zona radiata in the early growing phase and later, when there is no zona radiata at the circumference of the ovum.

The pre-ovulatory granulosa cells have the characteristics of protein

forming cells with abundant Golgi material and endoplasmic reticulum and numerous mitochondria showing close packed cristae. The post-ovulatory granulosa cells are quite strikingly different from the pre-ovulatory cells with their aggregation of large dense granules and distended spaces from which probably the lipid has been extracted.

Precise details of the pathways of biosynthesis of oestrogens are still not well defined in mammals and there is even less information available with regard to avian metabolism. The accepted routes of steroid biosynthesis established by various groups of workers (Lipsett and Hökfelt, 1961; Mulrow, Cohn and Kuljian, 1962; Weliky and Engel, 1962) are summarised in Fig. 1.

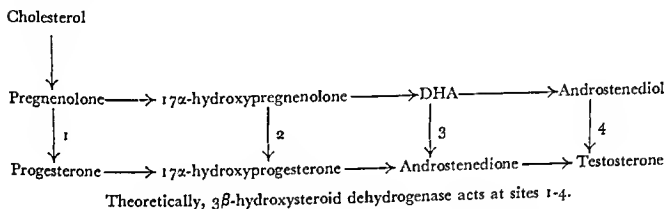


FIGURE 1

The histochemical results obtained with the free steroids suggest that the granulosa cells and theca interna cells of the fowl are capable of carrying out conversions 1, 3 and 4 readily, provided that the appropriate isomerase (Krüskemper, Forchielli and Ringold, 1964; Ewald, Werbin and Chaikoff, 1964) is present. It thus seems likely that progesterone is produced, though whether it is released or undergoes  $17\alpha$ -hydroxylation and subsequent conversion to oestrogens is not known. Androstenedione and testosterone are unlikely to represent the end products of conversions 3 and 4 and it is reasonable to suggest that they are intermediates in the biosynthesis of oestrogens, but it must be pointed out that the present histochemical results give no information concerning the other enzymes involved in these biosynthetic pathways. The relatively poor utilisation of  $17\alpha$ -hydroxypregnenolone suggests a degree of enzyme-substrate specificity and resembles the situation in the mouse testis (Baillie and Griffiths, 1964) and in foetal human testis (Baillie, Niemi and Ikonen, 1965).

$3\beta$ -Hydroxysteroid dehydrogenase activity is histochemically demonstrable in the theca interna of the human ovary (Ikonen, Niemi, Pesonen and Timonen, 1961; Deane, Lobel and Romney, 1962) and the rat ovary (Levy, Deane and Rubin, 1959; Taylor, 1961; Rubin, Deane, Hamilton and Driks, 1963) and the results obtained in the present avian material accord with the mammalian findings. The situation regarding whether this enzyme is histochemically demonstrable in the granulosa cells is less satisfactory. Levy *et al.* (1959), Deane

*et al.* (1962) and Rubin *et al.* (1963) consider that  $3\beta$ -hydroxysteroid dehydrogenase activity is histochemically demonstrable only in the granulosa cells of atretic follicles. Niemi and his co-workers (personal communication) have noted  $3\beta$ -hydroxysteroid dehydrogenase activity in the granulosa cells of apparently healthy human follicles and Ferguson (1965) in this laboratory has found histochemically demonstrable  $3\beta$ -hydroxysteroid dehydrogenase in the granulosa cells of normal rat, mare and human follicles. The present avian results support the views of Niemi and his co-workers cited above and those of Ferguson (1965), but the species difference must be borne in mind.

The precise metabolic role of the steroid sulphates with respect to steroid hormone biosynthesis remains unknown. Several recent reports of direct steroid sulphate metabolism have recently been published. Of particular interest are the conversions: (1) pregnenolone  $^3\text{H}$  sulphate- $^{35}\text{S}$  to  $17\alpha$ -hydroxypregnenolone- $^3\text{H}$  sulphate- $^{35}\text{S}$  *in vitro* using hyperplastic adrenal homogenates (Calvin and Lieberman, 1964); (2) pregnenolone sulphate- $^{35}\text{S}$  to DHA sulphate  $^{35}\text{S}$  *in vivo* (Calvin, Vande Wiele and Lieberman, 1963); and (3) cholesterol- $^3\text{H}$  sulphate- $^{35}\text{S}$  to the radioactive DHA sulphate *in vivo* (Roberts, Bandi, Calvin, Drucker and Lieberman, 1964). In each instance, conversion occurred without cleavage in the ester group.

The histochemical results obtained with  $3\beta$ -sulphoxy derivatives of pregnenolone,  $17\alpha$ -hydroxypregnenolone and DHA are different from those obtained with the free steroids. Of particular interest is the poor histochemical reaction observed using DHA sulphate which contrasts markedly with the rapid deposition of formazan obtained with the free steroids. On the basis of similar findings in the Leydig cells of the mouse testis (Baillie and Griffiths, 1965) and foetal human testis (Baillie, Niemi and Ikonen, 1965) it has been suggested that the presence of a  $3\beta$ -sulphoxy group on the steroid molecule might affect the ability of the substrate to bind with the  $3\beta$ -hydroxysteroid dehydrogenase. This might explain the enhanced utilisation of  $17\alpha$ -hydroxypregnenolone sulphate and the poor utilisation of DHA sulphate. Solubility factors and membrane permeability effects, however, cannot be excluded in this histochemical system.

Pregnenolone acetate and DHA acetate have been shown to be satisfactory substrates in this histochemical reaction in the human adrenal and placenta (Baillie, Cameron, Griffiths and Hart, 1965) and their ready utilisation by this tissue was not unexpected. The biological significance of these steroids, if any, is at present unclear. Of especial interest is the utilisation of cholesterol acetate by the theca interna and granulosa of the youngest follicle studied and the failure of older follicles to use this substrate. This changing utilisation of cholesterol acetate with the age of the endocrine tissue closely resembles Hart's (unpublished observations) finding in the developing human placenta and further work is required to clarify its meaning.

The ability of the theca interna and granulosa to convert cortisol to cortisone implied by the presence of  $11\beta$ -hydroxysteroid dehydrogenase, accords with the distribution of this enzyme in the rodent ovary (Baillie *et al.*, 1965a) and is of interest. The significance of the presence of  $17\beta$ - and  $20\beta$ -hydroxysteroid dehydrogenases is less clear but the existence of four steroid dehydrogenases in the theca interna and granulosa points unmistakably to a steroid biosynthetic function for these tissues.

It might seem to be overrating the functional capacity of granulosa cells to suggest they are responsible for the secretion of the ovarian hormones. What has been demonstrated histochemically, however, is that they are equipped with the enzymes necessary to produce the hormones and not that they are in fact producing them at any one time.

In the fowl the ovarian hormones are probably mainly concerned with the activity of the different segments of the oviduct and may well be produced in the pre-ovulatory phase by the so-called luteal cells of the theca interna. The granules of the post-ovulatory granulosa cells certainly represent some form of secretion—most probably oestrogen and/or progesterone, correlating in time with the passage of the ovum down the oviduct.

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# 6

## BEHAVIOUR OF SPERMATOOZOA IN THE OVIDUCT IN RELATION TO FERTILITY

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### *Synopsis*

SPECIAL requirements for fecundity in birds, imposed by the sequential nature of ovulation, are met by the presence of sperm-storage glands in the oviduct, in which the functional capacity of spermatozoa is prolonged, and from which spermatozoa are released, a few at a time by an unknown mechanism, in synchrony with ovulation.

Sperm-storage glands are present in the caudal infundibulum and in the uterovaginal junction; the latter are probably the normal site of sperm storage after copulation or intravaginal A.I. Storage of spermatozoa in infundibular glands may be accomplished through depositing semen anterior to the uterovaginal junction. Fertilisation by such spermatozoa results in an abnormally large number of early embryonic deaths.

### *Introduction*

The sequential nature of ovulation in birds imposes some special requirements on the reproductive process, requirements which are met by a set of mechanisms for prolonging the life of spermatozoa in the oviduct and protecting them from being swept out by successive descending eggs. The paramount importance of these mechanisms for fecundity in domestic species has led to a research project on them at Davis. Some interim results are the subject of this paper. The project has been a collaborative effort by Dr F. X. Ogasawara and the author; most of the experimental results described have been those of two graduate students, now Drs L. W. Bobr and H. P. Van Krey.

### *Results and Discussion*

In both natural mating and artificial insemination (A.I.), spermatozoa are ordinarily deposited in the mid-vagina. In copulation the hen everts the distal vagina and the cock, having no intromittent organ, deposits the semen on the exposed portion. Even with an artificial intromittent organ (e.g. a syringe), penetration is ordinarily limited to

the sigmoid loop of the vagina. From thence spermatozoa reach the uterovaginal junction, probably by their own motility, and from this point some are carried rapidly and passively the length of the oviduct, but apparently very few actually reach the site of fertilisation in the infundibulum. At least, very few are ordinarily found there. All spermatozoa disappear from the oviduct lumen, usually within 24 hr. of being deposited, although eggs continue to be fertilised for 2 weeks or more without remating or further A.I., so that a residence site other than the oviduct lumen must exist for spermatozoa.

Van Drimmelen (1946) believed he had found this residence site in the so-called "sperm nests", which are tubular glands in the caudal infundibulum. He found large numbers of spermatozoa in these glands, in a single hen 8 days after intraperitoneal A.I., and occasional spermatozoa in others after natural mating. Since we rarely found any spermatozoa in these glands we concluded that another residence site must exist for spermatozoa in the oviduct. Dr P. E. Lake called our attention to spermatozoa in glands of similar appearance in the uterovaginal region. Bobr (1962) and Bobr, Lorenz and Ogasawara (1964) investigated these glands and concluded that they are the normal residence site of spermatozoa after natural mating or intravaginal A.I. Immediately after such introduction a large number of spermatozoa enter the uterovaginal glands, and at least some spermatozoa can be found in these glands until the end of the fertile period. The infundibular glands, in contrast, are entered by spermatozoa under these circumstances only rarely and only in small numbers.

The question immediately arises, how can spermatozoa in uterovaginal glands serve to fertilise eggs? Bobr, Ogasawara and Lorenz (1964) discovered that small numbers of normal-appearing spermatozoa could be found free in the oviduct lumen immediately before or immediately after ovulation. If the examination of the bird was delayed until 2 or 3 hr. after ovulation, however, spermatozoa in the lumen were very few in number and showed evidence of degeneration. It appears that a few spermatozoa are released from the uterovaginal glands at about the time of ovulation, and ascend rapidly to the fertilisation site in the infundibulum. Those that do not reach the ovum have a very short remaining life-span in the oviduct lumen.

The mechanism of release is still unknown. Van Krey (1964) and Van Krey, Ogasawara and Lorenz (1964) investigated this problem and ruled out a number of possible mechanisms. Spermatozoa are always pointed towards the blind ends of the glands, so it seems unlikely that they could swim out. No muscular tissue or myoepithelial cells have been identified, so it appears unlikely that spermatozoa could be extruded by glandular contraction. Grigg (1956) postulated that the stretching effect of the passing yolk causes release of spermatozoa from infundibular glands; however, the oviduct he examined was only 3 hr. post-A.I., and spermatozoa should have still been free in

the lumen. Studies at Davis have suggested that such distension of the infundibulum dislodges spermatozoa only from superficial folds, and Dr Van Krey was unable to identify released spermatozoa in the uterovaginal region following simulated oviposition. He did find them after hormonally induced oviposition, but in smaller numbers than after normal oviposition, and hormones failed to release spermatozoa unless ovulation or oviposition was actually induced. He concluded that sperm release is a result of a complex of stimuli associated with the entire oviposition-ovulation sequence. The release mechanism itself is still unknown. The possibility remains that spermatozoa may be flushed out by secretion, but, although secretory material has been observed in these glands, no secretion pattern has been associated with events of the reproductive cycle.

The biological significance of the infundibular glands remains less well understood than that of the uterovaginal glands. Although spermatozoa do not enter them in significant numbers during the normal reproductive process, following special techniques (see below) they may harbour large numbers, and spermatozoa in infundibular glands are released in association with the ovulation cycle much like spermatozoa in the uterovaginal glands (Bobr, Ogasawara and Lorenz, 1964). In order to compare the sperm-storage and sperm-nurturing capacities of the two sets of glands, it was necessary to compare fertility patterns resulting from spermatozoa stored in one set or the other. This could be done only by filling one set while leaving the other empty.

Mid-vaginal A.I. was employed to fill the uterovaginal glands while leaving the infundibular glands nearly or entirely empty, and additional hens were subjected to intrauterine A.I. to fill both sets of glands (Bobr, 1962; Bobr, Lorenz and Ogasawara, 1964). Used for the latter insemination was a technique recently developed in Davis (Bobr, Lake, Lorenz, Ogasawara and Krzanowska, 1965) whereby prolonged but gentle pressure exaggerates the eversion of the vagina until the uterovaginal junction is exposed and may even be somewhat dilated. The uterus can thus be penetrated without the trauma and interference with egg production described by Allen and Bobr (1955). Hens with spermatozoa in the infundibular glands but not in the uterovaginal glands were, at first, more difficult to prepare. Bobr (1962) had described a single hen in that condition following intraperitoneal A.I., but in our hands this technique has given results too uncertain and erratic for experimental use. Several attempts to destroy the uterovaginal glands by surgery or electrocautery were unsuccessful. However, Van Krey (1964) and Van Krey *et al.* (1964) made the important discovery that spermatozoa introduced into the midmagnum via laparotomy and oviduct puncture travelled anteriorly only, engorging the infundibular glands with tremendous numbers while the uterovaginal glands remained empty (see also Van Krey, Ogasawara and Lorenz, 1965).

A.I. into the magnum via laparotomy slowed egg production



temporarily, but not enough to interfere seriously with determination of the resulting fertility pattern. This pattern was strikingly and unexpectedly altered. On the one hand, the average fertility span was prolonged significantly, to nearly 19 days, compared to only 13 days by intravaginal or intrauterine A.I.; and, on the other hand, the initial fertility as determined by examination of blastodiscs incubated 24 hr. was significantly reduced, to less than 48 per cent during the first week, compared to 89-92 per cent by other techniques. This result not only was unexpected but was completely new; simultaneous lowering of the fertility level and prolongation of the fertility period has not been observed previously.

Such an abnormal fertility pattern, and particularly the low *initial* fertility, suggested the possibility of an abnormally high incidence of pre-oviposital embryonic mortality, and, indeed, this proved to be the case. In a follow-up experiment in which eggs were broken without incubation and apparently infertile or doubtful eggs were subjected to the Kosin test (Kosin, 1944), the first and second week fertility estimates were increased strikingly, respectively to 75 and 87 per cent, but the first-week fertility was still below that with other methods of A.I. Consequently, in the next experiment, apparently infertile blastodiscs were sectioned and stained for microscopic examination; considerable numbers proved to have undergone cellular division, but had died too early to be detectable by the Kosin test. Four of five birds so checked were 100 per cent fertile during the first 10 days, yet only 20 to 75 per cent of the fertile eggs were detectable by gross visual examination of unincubated blastodiscs. The fifth hen had 33 per cent apparent fertility and twice as many truly fertile eggs. In a more complete experiment, the very early embryonic death was shown to occur more frequently in eggs laid during the first week after intramaginal A.I. than during the second. Thus, the embryonic mortality pattern is also paradoxical; in all previous observations, more embryos died, and died younger, as the post-insemination period increased.

The paradoxical fertility and embryonic mortality patterns after intramaginal A.I. doubtless have biological explanation but discussion of their natures must at present be speculative. The prolonged fertility might be evidence of superiority of the infundibular glands, but with the semen doses used the glands were engorged with tremendous numbers of spermatozoa, so that the prolongation of fertility may have been simply due to the presence of such large numbers. Such large numbers close to the site of fertilisation may also have been responsible for the excessive embryonic mortality; at least the possibility must be considered that polyspermy, resulting in abnormal embryos, may be responsible for at least some of the embryonic deaths. If so, the normal function of infundibular glands may be to remove excess spermatozoa from the site of fertilisation and reduce the probability of polyspermy.

An alternate hypothesis might be that the functional normality of spermatozoa is less well maintained in infundibular structures than in uterovaginal glands, especially, perhaps, in the numerous crypts and folds that become as engorged with spermatozoa as the actual glands after intramaginal A.I.

A third alternative is that a sperm-selective mechanism may be operative in uterovaginal but not in infundibular structures. Allen and Bobr (1955) reported, for example, that spermatozoa handicapped by admixture with glycerol fail to fertilise after being deposited in the vagina, but produce some fertility (with unknown embryonic consequences) if deposited in the uterus. An investigation, just completed (Ogasawara, Lorenz and Bobr, 1965), of the fertilising capacity of semen from cocks of very low fecundity also bears on this question. When inseminated intravaginally this semen fertilised very few eggs, but nearly all of these few hatched. When insemination was into the uterus, fertility was considerably increased, but nearly all of the increase was accounted for by pre-oviposital dead embryos.

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## THE ORIGIN AND COMPOSITION OF FOWL SEMEN

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### *Synopsis*

THE SOURCES of seminal plasma in the reproductive tract of the fowl, *Gallus domesticus*, have been discussed and the ionic composition of the fluid of the vas deferens has been established. This knowledge is useful in determining how the osmotic pressure and the pH conditions are produced prior to ejaculation. As the spermatozoa survive in the vas deferens for a much longer time than can at present be achieved *in vitro* it is considered that this fluid is a favourable physiological medium for the survival of the spermatozoa, hence the knowledge of its composition could serve as a basis for future studies on the development of semen diluents.

The transient appearance of fragments of spermatid cytoplasm in the structure of the spermatozoon is described and discussed.

### *Introduction*

Notwithstanding the variation between mammalian species, there are obvious differences between the fowl and mammal in the general anatomy and physiology of reproductive organs and reproductive functions. Unlike the mammal, the male domestic fowl, *Gallus domesticus*, does not possess accessory reproductive organs, e.g. seminal vesicles, prostate gland, Cowper's glands or glands of Littré; the epididymis is extremely short; the testes are internal, are of soft consistency and produce spermatozoa at a high body temperature (40°C.); within the body of the male the spermatozoa are stored in the extensive vasa deferentia; there is no penis, but several erectile structures (white body, round folds, lymph folds) are present in the mid-ventral and latero-ventral parts of the proctodaeum of the cloaca.

Much information is available on the chemical composition of mammalian semen, and on the significance of most of its constituents in the functioning of the spermatozoon and reproductive tract (Mann, 1964). The composition of fowl semen differs from that of the

mammal in several respects, due partly to the absence of the common accessory reproductive organs found in the mammal and thus of their typical chemical secretions. The spermatozoa of the fowl mature and survive in the vas deferens for a much longer period than they can at present be stored *in vitro*. For several years investigations have been made on the nature of this environment in which fowl spermatozoa live prior to and immediately after ejaculation. A knowledge of the chemical composition of the fluid of the vas deferens could provide useful information not only on the basic ionic environment consistent with the survival of spermatozoa, but also on possible metabolic processes associated with the functions of the spermatozoa and the reproductive tract. Metabolic substrates, intermediary metabolites and end-products of metabolism will all be included in the fluid. A large amount of information is available in the literature appertaining to the metabolism of several types of normal and abnormal cells of a wide variety of body organs in both mammals and birds. It would be useful to know how much of this information can directly explain the function of spermatozoan cells. However, until more information is available about their composition and that of their immediate fluid environment it is impossible to assess how far we are justified in using the information on general cell metabolism either to develop the techniques of storage of semen, or to explain fully any of the normal or abnormal functions of the spermatozoa in reproduction. Also when considering the chemical composition of the fluid of the vas deferens, due regard must be paid to the evolutionary history of the urinary and reproductive systems, and the possibility that the vas may still retain some of its vestigial functions as an excretory organ.

#### *Sources of seminal constituents in the reproductive tract*

A drawing of the reproductive tract of the male fowl is shown as Fig. 1. The main role of the testis is to produce spermatozoa. Nevertheless, the active testis of the fowl is of soft consistency and hence the possibility of the fluid of the seminiferous tubules contributing significantly to the composition of the semen of the fowl remains to be investigated.

The system of tubules comprising the rete testis, vasa efferentia and epididymis form a small, elongate mass lying on the hilum of the testis. This region is often referred to as the epididymal region. The epididymis, unlike that of the mammal, is very small and is merely a short collecting tube from which the spermatozoa and other fluid constituents enter into the vas deferens, which forms the largest part of the tubular reproductive tract of the male fowl. Histologically, there is evidence of much holocrine and apocrine secretory activity by the lining epithelium of the entire duct system (Lake, 1957a); thus fragments of cells as well as secretory products of intact cells are shed into the seminal

plasma. The spermatozoa are stored in the vas deferens, a function which in mammals is subserved by the extensive epididymis, and thus the possibility of a continuous exchange of chemical constituents between spermatozoa, fluid environment and epithelial cells must be considered when assessing the composition of the fluid of the vas deferens from the results of biochemical analyses.

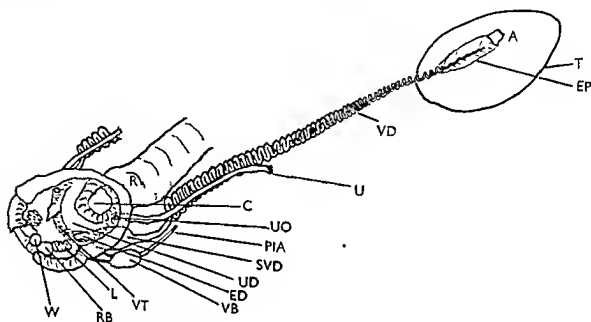


FIG. 1. Drawing of the reproductive tract and cloaca of the male fowl, *Gallus domesticus*. Right side in situ and dorsal part of cloaca removed.

A: adrenal gland. T: testis. EP: epididymal region. U: lower portion of the ureter. UO: opening of ureter into cloaca. R: rectum. C: coprodaeum. VD: vas deferens. SVD: sac-like ending of vas deferens. ED: ejaculatory duct projecting into cloaca. VT: fold of vascular tissue forming border of urodaeum and proctodaeum. UD: urodaeum. VB: internal vascular body (Gefäßreicher Körper). PIA: internal pudendal artery. L: erectile structures in the proctodaeum, i.e. lymph fold. RB: round fold. W: white body.

There is a dilated, sac-like, distal portion of the vas deferens, but there is no evidence to suggest that it is analogous to the secretory ampulla of mammals.

In the mid antero-ventral part of the proctodaeum of the cloaca is situated a structure called the *white body* which is flanked by *round folds*; slightly posterior to the *round folds*, on either side, are the *lymph folds*. These structures together with a fold of vascular tissue surrounding the fore part of the urodaeum erect during copulation. The mechanism of erection has been studied by Nishiyama (1955) and Nishiyama and Ogawa (1961). Lake (1957a) agreed with their observations except that the inflow of blood to some of the tissues was also considered to contribute to the processes of erection. In fact, whole blood can sometimes be squeezed through some of the epithelial surfaces during the artificial collection of semen. Slightly postero-dorsal to the *lymph folds*, and protruding into the urodaeum, are the ejaculatory ducts which are

the external extensions of the vasa deferentia. During copulation these become engorged with the contents of the vasa deferentia that are ultimately vigorously ejected into the hen's vagina.

The whole semen of mammals is defined as the spermatozoa and fluids that are ejected from the penis. Some fluid is derived from the vas deferens, but the bulk of it is produced by the various accessory reproductive organs that either open into the anterior urethral portion of the penis (seminal vesicles, prostate gland and Cowper's glands) or are scattered along the penile urethra (glands of Littre). The definition of fowl semen is not clear. A transparent fluid, in some respects similar in chemical composition to a dialysate of blood plasma (Nishiyama, 1957), is easily expressed through the epithelial surface of the erected lymph folds during the artificial collection of semen. Also a smaller volume of fluid of similar composition is obtained from the erectile fold of vascular tissue which is at the boundary of the urodaeum and proctodaeum. These fluids clot in the same way as blood plasma and they are inimical to the survival of spermatozoa (Nishiyama, 1951, 1952; Lake 1956). Whether or not they can be considered as accessory reproductive fluids remains in doubt (Lake, 1957b). They are so regarded by Nishiyama and Fujishima (1961) on the evidence that if a plastic collector is strapped over the cloaca of a cock during natural copulation some transparent fluid is ejected into the collector. Lake (1957b) could find no evidence of the presence of transparent fluid in the vagina of hens after natural copulation and suggested that the true semen of the fowl was that material ejected from the ejaculatory ducts, i.e. spermatozoa together with fluid accumulated in the vas deferens. Further studies are required before an unequivocal definition of fowl semen and the significance of the cloacal glandular structures can be established. The amount of transparent fluid which is obtained during the artificial collection of semen is very variable between individual males, and in the same males at different times. In some breeds of the domestic fowl and in certain species of birds no transparent fluid is obtained at ejaculation with the contents of the vas deferens.

*Composition of the fluid of the vas deferens and the blood plasma*

The ionic composition of the fluid of the vas deferens provides an indication of the principal ions contributing to a specific, isotonic medium in which spermatozoa are able to carry out their fundamental metabolic processes.

A comparative analysis of freshly collected seminal plasma (vas deferens fluid) and the blood plasma of the Brown Leghorn cock has recently been published by Lake and El Jack (1961). Their results, together with other analyses on males of the same flock, are summarised in Table 1. The carbon dioxide content of the respective fluids was estimated with a Beckman gas chromatograph in the following manner.

Blood or semen (less transparent fluid) was collected under liquid paraffin in the cold and immediately centrifuged (2,000 g; 20 min.) at 4°C. The seminal fluid or blood plasma was introduced into the chromatograph, taking the usual precautions to avoid loss of CO<sub>2</sub> to the atmosphere. CO<sub>2</sub> was released and estimated; a calibration of the instrument had been performed previously, using standard sodium carbonate solutions. An examination of Table 1 shows that although the totals of cations are equal in the respective fluids, the calcium

TABLE 1

*Average content (mEq/100 ml.) of the principal ions in the blood and seminal plasmas of the domestic cock*

Ion	Blood	Semen
Ca <sup>++</sup>	0.6	0.2
Mg <sup>++</sup>	0.2	0.5
Na <sup>+</sup>	17.0	16.1
K <sup>+</sup>	0.6	1.3
	18.4	18.1
Cl <sup>-</sup>	12.1	3.7
HPO <sub>4</sub> <sup>-</sup>	0.4	0.2
HCO <sub>3</sub> <sup>-</sup>	2.6	2.0
Pyruvate	0.04	0.03
Lactate	0.54	0.4
2-oxo-glutarate	0.01	0.14
Glutamate	0.02	7.3
Creatine	—	0.7
	15.7	14.5

concentration is lower, and that of magnesium and potassium higher, in the seminal fluid. A most interesting fact about the composition of the seminal fluid is that the glutamate ion assumes the role of chloride in blood plasma as the principal anion. Another interesting feature is the presence of a relatively high quantity of 2-oxo-glutarate which is possibly associated with the high glutamic acid concentration. There is a small anionic deficit of both fluids. The amount of protein is low in the seminal fluid (0.8 per cent) compared to blood plasma (5 per cent), and if the quality of the proteins were similar to that of human blood plasma, only another 0.3 and 1.8 mEq of cations per 100 ml. respectively could possibly be neutralised by anionic groups of proteins. In spite of the anionic deficit for seminal fluid the total content of all ions (32.02 m-moles per 100 ml.) corresponds to the freezing point depression,  $\Delta$ -0.593°C., which has been measured (Lake and El Jack, 1964). Thus the major seminal constituents have been accounted for in the analyses. Some of the anionic deficit may be more apparent than real, because it is possible that some of the magnesium and potassium (cations) are derived from the breakdown of spermatozoa in the vas deferens. This problem is being investigated.

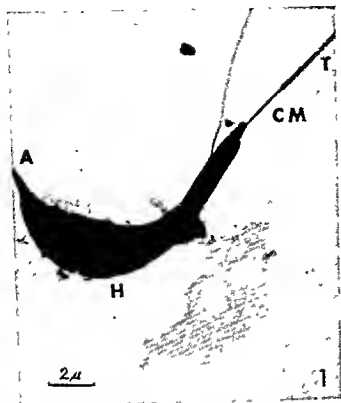


FIG. 1. Late spermatid stage taken from the seminiferous tubule. The cytoplasm has not yet been shed and the mitochondria have not formed on the middle piece. Acrosome, A; filament core of middlepiece, CM; filaments already covered by tail sheath, T; head of sperm still enclosed by spermatid cytoplasm, H.

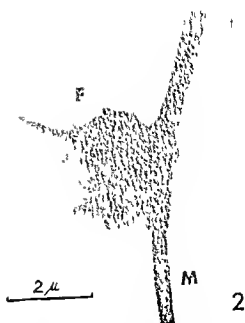


FIG. 2. Spermatozoon from the seminiferous tubule. Large fragment of spermatid cytoplasm at caudal end of head, F; middlepiece with mitochondrial sheath and membrane formed around the filaments, M; part of head, H.

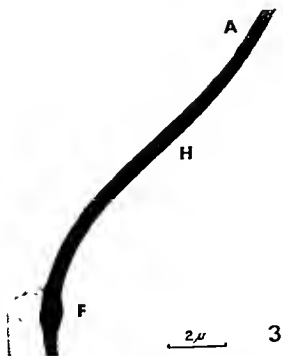


FIG. 3. Spermatozoon taken from the rete testis tubules—vasa efferentia region. Small fragment of spermatid cytoplasm still visible, F; Head, H; acrosome, A.

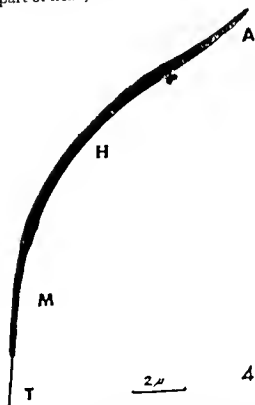


FIG. 4. Another spermatozoon from the epididymal region showing no obvious sign of possessing a spermatid fragment. Tail, T, middlepiece, M, head, H, acrosome, A.



*The structure of fowl spermatozoa on release from the testis and in the proximal ducts of the reproductive tract*

The mammalian spermatozoon possesses a *kinoplasmic droplet* on its midpiece whilst it is in the epididymis. This structure is at the base of the head when the spermatozoon is shed from the seminiferous tubule and it migrates caudally along the midpiece as the spermatozoon moves to the tail of the epididymis. Normally it is shed in the vas deferens. The migration of the *kinoplasmic droplet* is said to be associated with a "ripening" process which occurs in mammalian spermatozoa. Some authors regard it as a remnant of spermatid cytoplasm, others believe that it specifically nourishes the spermatozoon during its passage down the epididymis (Mann, 1964); its exact significance is still a moot point.

A droplet has never been reported in avian spermatozoa. Recently, in an electron microscopical examination of whole mounts of spermatozoa taken from various regions of the reproductive tract of the male, it was observed that free spermatozoa taken from squashes of the testis possessed variable-sized fragments of spermatid cytoplasm adhering to the caudal end of the head. These were barely visible when the spermatozoa were taken from the vasa efferentia-epididymis region of the tract (Plate 11, Figs. 1, 2, 3, 4). It is not possible at present to say whether this cytoplasmic fragment is analogous to the *kinoplasmic droplet* of mammals. If it were associated with maturation then its transient appearance in the bird might indicate that the spermatozoon of the bird matures very rapidly and, as a result may be one of the contributing factors to the difficulties encountered in storing fowl semen *in vitro*. This possibility has yet to be investigated.

### *Discussion*

It is often said that the seminal plasma of man and other mammals is an unfavourable medium for the prolonged survival of spermatozoa *in vitro*. An examination of the composition of seminal plasma in these cases would not therefore be expected to contribute significantly to the development of synthetic media for survival *in vitro* of spermatozoa. On the other hand, the harmful conditions may be produced when the accessory secretions are added at the time of ejaculation. Haag (1959) described an adverse effect on stallion spermatozoa of a high content of sulphhydryl compounds in the accessory secretions. Rozin (1961) showed that the fertilising capacity of spermatozoa from certain infertile human donors could be restored if they were separated from their own seminal plasma and resuspended in cell-free seminal plasma from proven fertile donors.

Components of the secretions of the accessory reproductive glands are believed to have a stimulating action on spermatozoa which would tend to shorten their viability *in vitro* (Huggins, 1945). Investigations

with the fowl have been designed to find out the ionic composition of the fluid in the vas deferens which determines the osmotic conditions and pH in which spermatozoa can retain their integrity. In the vas deferens the spermatozoa survive for much longer periods than can at present be achieved when stored *in vitro*. The fluid of the vas deferens is considered to be the seminal plasma of the fowl, until it can be shown that other fluids also enter the vagina at copulation.

Although the fluid of the vas deferens is a favourable physiological medium for spermatozoa it is not considered likely that each of its components is intimately concerned with metabolic functions of the spermatozoa themselves. It is quite clear that seminal plasma is not only a secretory product of intact cells but contains also fragments of cells from the lining epithelia of the reproductive tract. It is conceivable that the whole or part of the large amount of acid phosphatase present in the semen of the fowl originates from cell fragmentation (Bell and Lake, 1962; Lake 1962). It has been reported that much uric acid and urea is present in fowl seminal plasma (Klymiukowna, Olbrychtowa and Pytasz, 1960), and if these observations exclude the possibility of urinary contamination during semen collection, the role of the vas deferens as an excretory channel must be considered.

With a knowledge of the composition of the fluid of the vas deferens, which indicates the environment to which spermatozoa are conditioned, together with a similar study of the oviducal environment, in which spermatozoa again survive for prolonged periods, it might be possible to determine the optimal proper conditions for the functioning of spermatozoa. This information would be useful in investigations of infertility as well as for the development of synthetic diluents for the storage of spermatozoa *in vitro*. Finally, it must be pointed out that diluents, to be effective, must also be of such a nature that they do not disturb the environment in the oviduct where the spermatozoa are deposited, as otherwise their storage in the female would be impaired. This is an interesting problem for future investigation.

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# 8

## FERTILITY IN THE MALE IN RELATION TO NATURAL AND ARTIFICIAL INSEMINATION

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### *Synopsis*

FERTILITY problems in the male fowl associated with natural and artificial insemination are reviewed. The evaluation of the semen from individual cocks followed by the elimination of sterile birds and those with semen of poor quality has proved to be a practical way of improving fertility in artificially inseminated flocks. Two experiments using, respectively, 32 cocks and 120 cocks, showed a significant positive correlation between sperm motility and the percentage of fertile eggs (significant at the 1 per cent level in the first experiment and at the 5 per cent level in the second). Parallel experiments with flocks under natural mating conditions have shown that hens mated with cocks of high semen quality raised the percentage of fertile eggs by 5 per cent on the average, against the flocks of unselected cocks with average quality semen.

The relationship between thyroid activity on spermatogenesis has been discussed. Experiments on the effect of supplementation of iodinated casein of ascorbic acid to the diet, have shown a most significant effect of the diet supplemented with 200 ppm of ascorbic acid, by increasing the semen quantity and total spermatozoa per ejaculate. Addition of thyroprotein to 100 ppm ascorbic acid also seemed to increase the production of spermatozoa. Treatment with iodinated casein alone had a beneficial effect, mainly on the non-cellular fraction of the semen.

### *Introduction*

The economic importance of the individual male's fertility is much greater than that of the hen, because the male has the responsibility of fertilising a large number of eggs. The fertility of the cock is dependent upon many factors, some of which are already understood, while others are still obscure. There are some fertility problems in the male fowl which are associated with natural insemination, while others result from the practice of artificial insemination. In natural mating, the

fertilisation of the flock is closely related to the vigour of libido, which is itself controlled by hormonal and psychological factors as well as the social rank of the individual.

Many workers claim to have observed a significant correlation between libido and the fertility of the male (Wood-Gush and Alsbome, 1956; Craig, Cosida and Chapman, 1954). Others deny the existence of any such correlation (Kumaran and Turner, 1949; McDaniel and Craig, 1959). Ryan (1959) examined the sperm of some socially depressed males showing little sign of libido and found them to have a high fertilising capacity.

Some workers have recommended the use of aphrodisiacs (e.g. testosterone) in order to improve the fertility of the male. This idea is, however, at variance with our histological findings (Perek, Eckstein and Sobel, 1957). It was noted that, in cockerels, the beta gonadotrophic cells of the anterior lobe of the hypophysis disappeared significantly after testosterone treatment being replaced by chromophobic and thyrotrophic cells.

Inadequacies in flock management, such as an excessive number of males, unsuitable feeding troughs—or an inadequate supply of troughs—as well as heavy feathering around the vent are factors which might impair insemination and thus influence the fertilising capacity of the male in natural matings.

Problems of a different nature which might impair fertility are associated with the practice of artificial insemination. The artificial insemination of hens is not widely used on commercial farms, except in Israel. About 40 per cent of all hatching eggs produced in Israel are collected from artificially inseminated flocks. Despite the expense of the labour involved this method appears to be justified for the following reasons:

1. It permits the more efficient use of males of proven genetic stock, i.e. one male for an average of 40 females, instead of 1:10-15 in natural matings.

2. In general it ensures a higher fertilisation rate as reported by Cooper (1955) and by Lutzenberg and Doehl (1956).

3. It facilitates both the keeping of records and the control of egg production in breeding flocks and is the only system available for obtaining hatching eggs from a battery-kept flock.

On the other hand, there are limitations to the use of artificial insemination:

1. Milking of the semen requires special precautions: the use of clean and sterilised vessels is essential, and care must be taken to avoid faecal contamination of the sample. When expressing the semen from the males' papillae, care must be taken not to cause haemorrhage from ruptured blood capillaries, as this can considerably impair the fertility of the sample (Lake, 1957).

2. A suitable medium for dilution of the semen is not yet available, thus requiring the use of undiluted semen.

3. A sharp decrease in the fertility of the eggs has been observed after repeated insemination over a period of 3-6 months; the fertility of the eggs is sometimes decreased by as much as 50 per cent, and even lower figures have been noted. The cause of this decrease is as yet unknown and experiments should be undertaken to determine to what extent the male and female are individually implicated.

### *Experimental*

The measure of fertility, whether from natural mating or artificial insemination, is related to the general health of the males, the number of daylight hours, environmental temperature, the season of the year, to nutrition and to the qualitative and quantitative characteristics of the semen sample of each individual animal. The examination of semen from individual cocks with respect to its volume and quality, followed by the elimination of sterile birds and those with semen of poor quality, has proved to be a practical way of improving fertility in artificially inseminated flocks, and the same may hold true for flocks under conditions of natural mating.

Three controlled experiments have so far been carried out by members of our research staff (Snapir, Perek and Ackert, unpublished).

#### *Experiment 1 (Javneh)*

On one of the breeding farms, semen samples of 32 White Cornish cocks were examined for motility and sperm concentration and the samples were graded accordingly. The semen of each cock was used to inseminate 10-12 hens (White Plymouth Rocks). The percentage fertility of each batch of eggs was determined following incubation. A significant positive correlation was found between the gradings for motility of the sperm samples *in vitro* and the percentage of fertile eggs. It was shown that the correlation between sperm motility and the fertility of the hatching eggs was highly significant, as demonstrated by the results summarised in Table 1.

In order to exclude the "hen factor" from this experiment, insemination was suspended for more than 3 weeks, and the semen of the new cocks was again examined and distributed between the groups of hens. The latter experiment fully confirmed the highly significant correlation between sperm evaluation and the percentage fertility of the hatching eggs.

#### *Experiment 2 (Massuot)*

The purpose of this experiment was to repeat the experiments using a larger group (120) of cocks. This experiment confirmed the results obtained in the first experiment and showed positive correlation between

sperm quality and fertility (significant at the 5 per cent level). During the course of these experiments, many farmers, were trained to examine and evaluate semen samples and now apply their knowledge to improve fertility in their flocks.

### Experiment 3 (Bilu)

This experiment was designed to determine whether fertility in a naturally inseminated flock could be improved by evaluating the semen and eliminating the sterile birds or those with poor quality semen.

TABLE 1

*Correlation between sperm motility and percentage of fertile eggs*

Group of hens	Cock number	Motility grades*	Percentage fertility	Cock number	Motility grades*	Percentage fertility
A	1	50	88	17	45	5
B	2	45	44	18	10	0
C	3	50	90	19	60	72
D	4	40	9	20	75	80
E	5	35	13	21	35	55
F	6	55	23	22	85	90
G	7	40	0	23	50	10
H	8	50	60	24	85	95
I	9	65	54	25	80	64
J	10	95	90	26	90	61
K	11	20	0	27	40	18
L	12	0	0	28	62	62
M	13	30	0	29	82	90
N	14	10	20	30	85	60
O	15	95	54	31	87	95
P	16	35	46	32	60	57

Correlation coefficient (2)

+0.662†

+0.809†

\* From 0 to 100. † Significant at the 1 per cent level.

The cocks were placed in batteries for 10 days and after an initial period in which they were trained to ejaculate by the massage method, the samples were evaluated and the birds were divided into 2 groups, according to their gradings.

Cocks with only high quality semen were placed with hens in one pen. In another, the male birds formed a mixed group with only average quality semen. The numbers of hens and cocks were equal in both cases. The experiment was repeated several times. The final results showed a higher fertilisation percentage (5.23 per cent) in the eggs of the first group ( $P < 0.001$ ) (Table 2).

Seasonal variations in the fertility of the male have been observed by several investigators (Parker and McSpadden, 1913; Bonadonna and Pozzi, 1959; Perek and Snapir, 1963). These reports show an increase in sperm concentration and sperm volume during the spring months, and a decline from July to October. Some investigators believe that the decline in fertility results from a decrease in daylight.

Bajapi (1963) obtained an improvement in semen characteristics by extending the daylight hours and reported a rise in the percentage of motile spermatozoa and a decrease in the number of abnormal cells while the rate of motility was not changed significantly. The optimum photoperiod is reported to be 9-12 hr. daily (Furman, 1963) while 8 hr. light resulted in complete sterility of the male. It is possible that these findings might be due to a sudden change of the light rations rather than to the absolute period of illumination. Photoperiodic regulation in male birds would repay further study.

TABLE 2

*The effect of selecting cocks with high quality semen upon percentage fertility of eggs*

Test No.	Percentage fertility of eggs	
	Rooms with cocks with high-quality semen	Rooms with cocks with average-quality semen
1	88.3	82.2
2	88.0	85.3
3	86.7	82.6
4	85.7	80.0
5	84.5	87.8
6	92.8	81.0
7	94.5	82.3
8	95.0	83.9
9	90.0	88.9
10	87.7	86.1
11	93.3	90.0
12	91.6	85.6
13	91.1	85.5
Total average	89.9±0.95*	84.7±0.85*

\* Standard error of the mean.

Other investigators believe that the seasonal increase in environmental temperature is associated with a decrease in male fertility (Polge, 1950; Sacki, 1960). On the other hand, Kamar and Badreldin (1959) reported a beneficial action of the seasonal high temperature in Egypt on the semen quality of the Fayoumi breed. Perek and Snapir (1963) stated that the semen characteristics do not show a direct statistical correlation with the environmental temperature and may reflect merely a breed characteristic. The decrease in semen production observed in all the breeds during the autumn months may, therefore, be a result of reduced feed consumption during the summer, or the age of the birds, or may be due to reduced daylight.

Suddenly imposed stresses on cocks, such as the precipitate changes in temperature caused by the mid-eastern desert wind "hamsin", may likewise produce a temporary decline in semen quality 5-7 days after the imposition of the stressing agency (Perek and Snapir, 1963).



*Vitamins and Fertility in the Male*

The literature on this subject is sparse. Titus (1955) stated that hypovitaminosis A, induced in cocks at the age of 7 months, had no effect upon their fertility. On the other hand, Garcia and Parades (1957) reported that while libido remained normal there was a significant decrease in semen volume and sperm concentration in cocks fed a low vitamin A diet. When the mash was supplemented with 30,000 i.u. of vitamin A per kg. a significant improvement in the sperm characteristics was noted after 14 days.

Vitamin E-deficient diets, fed to cocks during 18 months, did not affect the fertility of the male (cited by Titus, 1955).

In recent years some investigations were carried out on the possible effect of ascorbic acid (AA) upon semen quality. Wawrzyniak (1956) found a stimulative effect of AA injections, on spermatogenesis and testes weight, in 70-day-old cockerels. Perek and Snapir (1963) reported that AA, when added as a supplement to the diet of White Plymouth Rock Males, had a beneficial effect upon semen volume and sperm concentration during the season of declining fertility. No statistical differences were obtained in motility between the AA treated and the control groups.

As a result of the report by Thornton and Deeb (1960) that AA in the chickens' diet influenced the birds' metabolism by increasing oxygen consumption. Perek and Lowe (unpublished), studied the effects of the addition of iodinated casein to the diet of AA-treated birds in order to test the effect of AA upon fertility and to ascertain, if possible, its mode of action.

Five treated and one control group were used, each consisting of 5 cocks (Fig. 1).

Group 1. Supplementation of 100 ppm AA to the diet.

Group 2. Supplementation of 100 ppm AA plus 0.015 per cent thyroprotein.

Group 3. Supplementation of 200 ppm AA.

Group 4. Supplementation of 200 ppm AA plus 0.015 per cent thyroprotein.

Group 5. Supplementation of 0.015 per cent thyroprotein.

Group 6. Unsupplemented control.

The experiment was started in June, at the onset of the hot season, and was continued until the middle of December. The results showed a most significant positive effect ( $P < 0.01$ ) of the diet supplemented with 200 ppm AA on group 3, increasing the volume of the semen and the total number of spermatozoa per ejaculate; sperm concentration was, however, somewhat lowered (Fig. 1). It can be stated therefore that AA supplementation affects the non-cellular fraction more noticeably than the cellular fraction.

At the 100 ppm level of AA (group 1) no statistically significant

influence on the volume of the ejaculate could be found. This group could be distinguished by its high concentration of spermatozoa, although the total number of spermatozoa per ejaculate did not differ from that of the control.

Treatment with thyroprotein alone had a beneficial influence on the non-cellular fraction, but it had a smaller effect on cellular concentration.

The groups receiving both AA and thyroprotein have shown little difference from those treated with AA alone, with respect to the quantitative characteristics of the semen. The addition of thyroprotein to the 100 ppm AA group's diet seemed to increase the cell production as well, so that the total spermatozoa per ejaculate was higher. Addition of thyroprotein to the 200 ppm AA group's diet caused a slight decrease in the total spermatozoa per ejaculate. This result could probably be explained by the report of Desmarais (1960) who stated that a small amount of thyroxine combined with a dose of ascorbate is equivalent in its action to a larger dose of thyroxine. It is thus possible that the combination of 200 ppm of AA with thyroprotein was excessive in this case.

The mode of action of AA in the diet on male fertility is not yet understood. Some investigators believe it to have a direct action upon the gonads, while others consider that it is mediated indirectly through

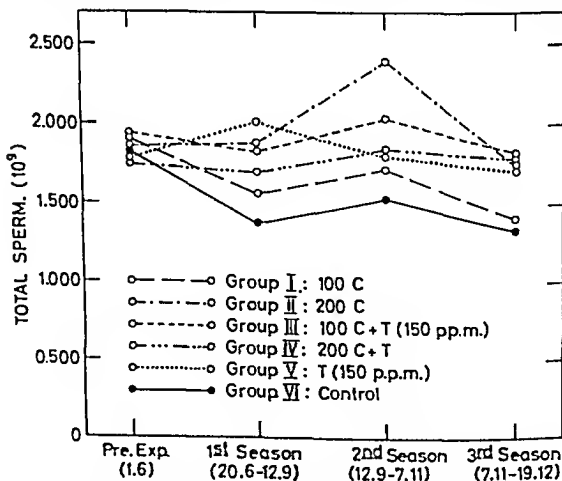


FIG. 1. The effect of Ascorbic acid (C) and of Thyroprotein (T) upon the total spermatozoa per ejaculate.

the thyroid (Desmarais, 1960) on the grounds that AA was shown to increase the metabolism of tyrosin and phenylalanine, which are respectively precursors of the adrenal and thyroid hormones. A relationship between thyroid activity and spermatogenesis in birds has been demonstrated by several investigators (Turner, 1959; Magsood, 1950; Shaffner, 1948) and it may be assumed that the process of sperm production and fertility are closely related to the body's general metabolism, but much further work remains yet to be done before this matter can be finally settled.

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## PART II

### METABOLISM AND NUTRITION

# THE ACCUMULATION OF WATER AND ELECTROLYTES IN THE EGG OF THE HEN

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## *Synopsis*

THE PROGRESSIVE accumulation of water, sodium, potassium, calcium and magnesium into the egg has been studied by analysing eggs sampled at various stages of development commencing with immature ovarian follicles. The accumulation of yolk substance is accompanied by a steady increase in water, calcium and magnesium contents until ovulation. Sodium entry, however, appears to dominate in the earlier phases of yolk formation giving way usually, but not always, to potassium dominance in the mature yolk. After ovulation no significant changes in content or concentration appear to occur in the yolk, at least until well after oviposition. This is despite the fact that, as the yolk moves down the oviduct, there are large changes in electrolyte concentration occurring in the developing white. In the magnum region the protein concentration is high and the overall electrolyte concentration is comparable to, but somewhat lower than that in blood plasma. By the time of oviposition, however, a large addition of water takes place accompanied by an approximate halving of the protein and overall electrolyte concentrations, with the notable exception of potassium, which may more than double in quantity in the final white. The greatest part of these changes occurs during the process of plumping when some forty per cent of the water of an egg is added without the apparent addition of the expected amounts of osmotically effective particles such as sodium ions. In both the yolk and white important amounts of potassium are added in the final stages of formation and there is evidence to suggest that this process can be curtailed. From the analysis of newly-hatched chickens it can be deduced that potassium deficiency could be the basis of some failures in hatchability.

## *Introduction*

About 70 per cent of an egg is water, but little attention has been paid to the way in which this important constituent is transferred from

the tissues of the hen to the yolk or white of the egg. Large movements of water, such as occur across the proximal tubule of the kidney or in exocrine secretions, are usually associated with movements of electrolytes, particularly sodium. Indeed, in those situations which have been most extensively studied the process initiating water movement is the active transport of an electrolyte such as sodium. These processes, and the chemical energy systems involved, have been the subject of much research in recent years. The fundamental papers in this field have been drawn together in an excellent monograph by Ussing, Kruhffler, Theysen and Thorn (1960).

The cells surrounding the ovum of the hen as it accumulates yolk and the cells of the oviduct involved in the manufacture of the egg white must be involved in the transport of considerable amounts of water and electrolytes. The object of the work described in this paper was to obtain an overall picture of the pattern of movements of water and electrolytes occurring at each stage from the initial growth of the ovum to completion of the fully formed egg.

The work began with an analysis of sequences of eggs from individual hens, with the object of discovering the end-point at which the processes occurring in the ovary and oviduct are aimed. However, the degree of variation from egg to egg within a sequence proved so great that the work, rather than answering the question, posed another: what is the electrolyte composition of the biologically ideal egg?

In an attempt to throw light on this problem, the electrolyte composition of a number of newly hatched chicks was determined, since the prime function of the egg is to produce a viable chick. Having thus obtained some knowledge of the end-point, the changes occurring *en route* were studied: analyses were made of the contents of a number of developing follicles and of egg yolks and whites obtained from oviducts either at operation or from birds killed at appropriate intervals after the estimated time of ovulation. The results were both surprising and more complex than had been expected. Nevertheless, the overall picture seems fairly clear; sufficient evidence will therefore be presented in this paper to establish the general sequence of events. Detailed consideration of the complex sequence of the accumulation of water and electrolytes in the egg will be left to subsequent communications.

### *Material and Methods*

The birds used were crosses between two (B and I) of the partially inbred lines of Brown Leghorns maintained at the Poultry Research Centre (Blyth and Sang, 1960). Birds with identification numbers prefixed M were hatched during June and July 1963, those prefixed N during January and February 1964. The material analysed consisted of: (a) follicles at various stages of maturity obtained from the ovary at operation; (b) eggs at various stages of development obtained from the

isthmus and magnum regions of the oviduct at operation or after death; (c) sequences of about 10 successive eggs from selected hens during their first year of lay; and (d) newly hatched chicks. In addition, 12 eggs collected at random from Thornber "404" birds in mid-lay were obtained from a commercial producer, for comparison with the eggs laid by the Brown Leghorns.

Shell, white and yolk were analysed for sodium, potassium, calcium and magnesium. Water content was estimated from the loss in weight after drying for 14 to 16 hr. at 100°C. The minerals were estimated from hydrochloric acid extracts of aliquots by atomic absorption spectroscopy (Willis, 1965); preliminary work showed that ashing in a muffle furnace was unnecessary.

### *Results*

#### *Yolk*

Table 1 sets out the absolute quantities of each constituent found, expressed in grams for dry matter and water, and in micromoles for the mineral content of each follicular ovum, white and yolk examined. The interest of the present research is directed towards the forces involved in the movements of water and ions in the various regions of the oviduct or across the cells of the follicular wall and hence the mineral results are expressed in moles rather than grams so that the nature of the various ionic concentration gradients can be readily appreciated.

The eggs were obtained in late September 1964 and the results presented are values obtained from 10 successive eggs laid in 14 days by bird M5273. The ten samples of eggs in the process of formation were collected from the magnum and isthmus regions of the oviduct. This material came from a mixture of N and M birds, and although the sources were not uniform the diversity of ages of the birds strengthens, rather than weakens, the conclusions drawn from the results.



TABLE I  
*Dry matter, water and mineral content of Brown Leghorn follicular ova, eggs and day-old chicks*

		Follicle				Oviduct eggs		Oviposited eggs		Day-old chicks	
		IV	III	II	I	I	10	10	6		
Number of samples		1	1	1	1	10	10	10	6		
Dry matter:	Yolk	1.3	3.7	6.0	8.4	10.1±0.3	10.4±0.5	10.4±0.5	—		
	White	—	—	—	—	2.7±0.2	3.7±0.1***	3.7±0.1***	—		
						12.8	14.1	14.1	8.8±0.3		
Water:	Yolk	1.1	3.1	5.0	6.9	8.1±0.1	8.8±0.4*	8.8±0.4*	—		
	White	—	—	—	—	12.2±1.1	27.3±0.4***	27.3±0.4***	—		
						20.3	36.1	36.1	24.8±1.0		
Na:	Yolk	50	115	180	185	255±38	214±22	214±22	—		
	White	—	—	—	—	1470±208	1860±93	1860±93	—		
						1725	2074	2074	1620±107		
K:	Yolk	25	125	185	235	225±50	240±20	240±20	—		
	White	—	—	—	—	130±20	660±37***	660±37***	—		
						355	900	900	980±27		
Ca:	Yolk	65	165	265	395	325±58	355±18	355±18	—		
	White	—	—	—	—	130±21	46±4***	46±4***	—		
						455	401	401	1850±98		
Mg:	Yolk	6	22	45	55	53±9	46±7	46±7	—		
	White	—	—	—	—	130±12	110±6	110±6	—		
						183	156	156	185±16		

Dry matter and water in grams; minerals in micromoles.  
 Significances are indicated by \* =  $P < 0.05$ , \*\* =  $P < 0.01$   
 and \*\*\* =  $P < 0.001$ . Means and Standard Errors.

TABLE 2  
Concentrations (millimoles/litre) of minerals in *Brown Leghorn follicular ova*, oviduct eggs and oviposited eggs

Significances are indicated by \* =  $P < 0.05$ ,  
\*\* =  $P < 0.01$  and \*\*\* =  $P < 0.001$ . Means and Standard Errors

Number of samples	Blood plasma †	Follicle IV	Follicle III	Follicle II	Follicle I	Oviduct eggs		Oviposited eggs	
						10	10	10	10
Na:	170	Yolk: 45 White —	37 —	36 —	26 —	32 ± 4.3 117 ± 8.6	25 ± 3.1 68 ± 3.8***	25 ± 3.1 68 ± 3.8***	25 ± 3.1 68 ± 3.8***
K:	6.3	Yolk: 22 White —	40 —	37 —	34 —	27 ± 5.0 10 ± 1.1	28 ± 3.3 24 ± 1.4***	28 ± 3.3 24 ± 1.4***	28 ± 3.3 24 ± 1.4***
Ca:	1.2 (Ca <sup>++</sup> ) 6.7 (Ca)	Yolk: 61 White —	53 —	53 —	57 —	39 ± 6.0 10 ± 1.4	42 ± 3.8 2 ± 0.3***	42 ± 3.8 2 ± 0.3***	42 ± 3.8 2 ± 0.3***
Mg:	1.2	Yolk: 5 White —	7 —	9 —	8 —	6 ± 0.9 11 ± 1.1	5 ± 0.6 4 ± 0.2***	5 ± 0.6 4 ± 0.2***	5 ± 0.6 4 ± 0.2***

†. The values for the mineral concentrations in blood plasma water are from cocks, with the exception of calcium.

The stability of the ionic concentrations in the yolk as it moves down the oviduct is a remarkable phenomenon because before ovulation there is a considerable passage of minerals into the yolk substance. Some idea of the way this occurs can be gained from an analysis of groups of follicular ova. Table 1 sets out the results obtained from the four largest follicles from hen M4170, which was a similar bird to M5273. It can be seen that in this series the absolute mineral content increases steadily as the yolk grows in size. In the smallest sample, considering sodium and potassium ions, the sodium ion is dominant. This appears to be a general rule for all developing ova up to about this size. Further development is usually accompanied by a slow reversal of this relationship, with the potassium ion dominating in the final product. This process of sodium and potassium ionic transport to the yolk appears to be rather labile because many yolks are found, both in eggs and in follicles, in which the ionic state does not correspond with the state of development indicated by the mass of yolk substance present.

There is a difficulty about taking a number of follicles and putting them into an age series based on weight alone because, even in an apparently regular layer, one cannot be certain that any particular follicle would ever have proceeded beyond the point at which it was sampled; atretic follicles being of such frequent occurrence. This may explain the finding in the present study of many irregular electrolyte sequences. However, regular sequences such as in M4170 are found where, as judged by the yolks found high up in the magnum region of the oviduct, it can be inferred that transport of sodium and potassium ions had proceeded regularly until at least fairly shortly before ovulation. Furthermore, the indications are that the proper course of development is such that in the mature yolk the potassium content dominates, and that this greater net entry of potassium relative to sodium occurs late in the maturation process.

The calcium content of yolks is surprisingly high, being comparable on a particle basis to the sum of sodium and potassium ions. The magnesium content is much lower but far from negligible. This could mean, on a charge basis, that the calcium and magnesium ions are rather important when considering the charge distribution across the yolk membrane. The actual state of both the calcium and magnesium has yet to be determined, hence the significance of the concentrations in available water cannot be assessed and the figures in Table 2 are inserted merely as a guide to the possibilities. However, even at 25 per cent ionisation the concentrations in the ovulated yolk are, on a charge basis, considerably greater than in blood plasma (Table 2).

The accumulation of calcium and magnesium by the growing yolk in the follicle appears to be appreciably more regular than in the case of potassium and sodium. Table 1 shows clearly how the calcium accumulation proceeds quite regularly in comparison to sodium and

potassium together. Table 2 shows that in the one set of follicles the concentration of calcium and magnesium have remained fairly constant while the potassium concentration has tended to increase and the sodium to decrease. This merely reflects the increasing potassium dominance previously referred to, and draws attention to the interesting possibility of a phase of actual exchange of sodium ions for potassium ions as the ovum enters the pre-ovulatory phase. Reference to the concentrations in blood in Table 2 indicates that at all stages of yolk formation shown there is an apparent uphill gradient for the transport of potassium, calcium and magnesium into the yolk substance and a pronounced downhill gradient for sodium. (There is not enough information to make any statement concerning the electrochemical gradients).

The analysis of follicular ova of mass less than a gram but greater than 100 mg. has so far proved difficult to interpret. Numerically they greatly exceed the five or six that are obviously on the way to maturity and it would seem that there are a number of stages where the mass remains fairly constant but composition is changing markedly.

### *White*

Table 1 shows that in contrast to oviposited and oviducal yolks there are big differences between the corresponding whites. The oviduct whites analysed would, if they had not been removed, have become incorporated in eggs of about the same final weight as those laid by M5273. In the oviduct, conditions varied from the magnum where no membranes are present and there is thus the possibility of more thick white being added, to the isthmus, where no membranes are present. But, despite the diversity of origin, two important points are apparent. In the first place, after it has passed through the isthmus the white gains a considerable amount of water and surprisingly too, a large amount of potassium. At the same time there is no significant change in the number of sodium ions present but there is a highly significant decrease in the quantity of calcium. On the other hand, the content of calcium in the white was found to be rather variable in comparisons both between birds and between breeds and for this reason any deductions based on this decrease must be tentative. A possible explanation is that some of the calcium in the white is involved in the initial processes of shell formation.

The fact that there is no significant change in sodium content may not be quite as straightforward as it appears. There is the process of plumping to be considered. From Table 1 it is clear that a large movement of water takes place into the white after it leaves the lower isthmus. From Table 2 it is apparent that in the final oviposited egg the concentration of sodium is almost halved, while that of calcium, because some has apparently left the system, is reduced by 80 per cent. This is consistent with a simple mechanism of dilution by the addition

of water, but despite the added water the concentration of potassium has more than doubled to reach a value about four times that found in blood plasma. Thus the critical phase is egg plumping, during which there is added to the egg some 40 per cent of its water or some 25 per cent of its final weight. It occurs after the membranes are formed around the white, but is already evident in the lower isthmus and is completed in the shell gland.

As judged by the final state, the water accumulated by the white is not accompanied by the expected amounts of osmotically effective particles such as sodium ions. Relative to blood, egg white, as judged by freezing-point-depression methods, is hypotonic. Cock plasma has an osmotic pressure of 320 milli-osmoles, whereas typical egg white in the present study had a value of some 260 milli-osmoles. The quantity of potassium moved into the white, as might occur in the process of plumping, is not sufficient to be osmotically important. As will be suggested in the next section this process may well have considerable biological significance. It is noteworthy moreover that such an important constituent of living cells is added so late in the egg forming process.

#### *Newly hatched chickens*

From the biological standpoint the egg is destined to produce a chicken. A chick to be viable must possess, among other factors, a certain mass of cells constituting the essential organs. As the internal milieu of all cells contains a high and carefully controlled concentration of potassium, the implication is that there is initially a definite minimal quantity of potassium present in the egg. Furthermore, apart from cellular water, the extracellular water must be sufficient to meet the needs of an adequate circulation and this necessitates at least the presence of an adequate quantity of sodium ions.

Table 1 sets out the total-body analysis of six newly hatched chickens and the mean quantities of minerals found can be compared with the total of the means in the whites and yolks of M5273. The importance of potassium in the chicken is indicated by the fact that the coefficient of variation is least for this mineral (6.8 per cent) followed by water (9.7 per cent), calcium (13 per cent), sodium (16.2 per cent) and finally, perhaps surprisingly, magnesium (20.8 per cent). On this basis it now seems probable that some of the eggs of M5273 were deficient in potassium. There was some potassium, about 200 micromoles, in the shells of the eggs from which these chickens were hatched, but it is questionable if much of this could be available to the developing embryo. The potassium content of the shell cannot be removed easily by simple leaching with water. It is interesting to note, however, how small is the absolute contribution of the shell to the calcium needs of the embryo, only some 60 mg. out of some 1,800 mg., and also that the shell might yield a small but possibly important quantity of magnesium to the chicken.

*Brown Leghorn and Thornber "404" eggs*

The Poultry Research Centre's Brown Leghorns are not very efficient birds by modern commercial egg-production standards, hence it was of interest to compare their eggs with some newly-laid Thornber "404" eggs taken at random from a commercial producer. These particular birds were in mid lay so that the eggs were comparable with those of M5273 already discussed. Table 3 sets out the results, giving the absolute quantities of dry matter, water and mineral content found

TABLE 3

*Egg weight, dry matter, water and mineral content of Brown Leghorn and Thornber "404" oviposited eggs*

Egg weight, dry matter and water in grams; minerals in micromoles.  
Significances are indicated by \* =  $P < 0.05$ , \*\* =  $P < 0.01$  and \*\*\* =  $P < 0.001$ . Means and Standard Errors

	Brown Leghorn	Thornber "404"
No. of Samples	10	12
Egg weight	59.2 ± 0.5	58.2 ± 1.1
Dry matter: Yolk	10.4 ± 0.5	9.2 ± 0.2*
White	3.7 ± 0.1	4.5 ± 0.2***
Water: Yolk	8.8 ± 0.4	7.9 ± 0.2*
White	27.3 ± 0.4	28.2 ± 0.6
Na: Yolk	214 ± 22	170 ± 10
White	1860 ± 93	1210 ± 63***
K: Yolk	240 ± 20	255 ± 14
White	660 ± 37	530 ± 28**
Ca: Yolk	355 ± 18	355 ± 24
White	46 ± 4	310 ± 28***
Mg: Yolk	46 ± 7	43 ± 3
White	110 ± 6	95 ± 5

in the whites and the yolks of the two sets of eggs. There is no significant difference between the mean weights of the eggs between the groups and, although not included in Table 3 there was no significant difference between the mean shell weights. As far as the yolks are concerned no significant difference was found in any of the mineral constituents. The yolks of the Brown Leghorns had just significantly more dry matter and water. Thus they were slightly bigger.

The "404" whites contained a very significantly greater quantity of protein than those of the Brown Leghorns, yet both whites had essentially the same water content. The sodium and potassium contents of the Brown Leghorn whites, on the other hand, were very significantly greater than those of the "404"s, which is rather surprising. The most interesting feature is, however, the very great difference in the calcium content. The "404"s had over six times as much calcium in the white. From Table 1 it can be deduced that the calcium and magnesium are added to the white in the oviduct, proximal to the final part of the isthmus, and from other evidence it is probable that they are both

added in the magnum region. It is interesting that the magnesium content seems to be relatively constant in the white (as it is in yolk also) whereas the calcium content is somewhat variable. Normally in other situations calcium and magnesium vary together, but in this situation there seems to be no consistent relationship. The significance of this finding is obscure.

The quality of the whites of eggs from the Brown Leghorns produced at the Poultry Research Centre is considered poor, and it is thus worthwhile to look for a correlation between the "quality" of egg white and its calcium content. Perhaps calcium is involved in the cross-linked ovomucin-lysozyme complexes postulated by Brooks and Halc (1961) to explain the mechanical properties of the thick white.

Although the fundamental significance of the differences in composition between the two breeds of birds cannot be usefully interpreted at present, it is nevertheless interesting that differences could be demonstrated with such small samples.

### *Discussion*

There are a number of features of great fundamental interest which arise from a consideration of the results of the ionic and water movements into the oviduct and then into the membraneous egg. In general it seems clear that a simple system of net transfer into the oviduct will not explain the findings. The functioning of the tubular cells of the nephron would seem to be a somewhat analogous situation, except that the oviduct in some respects is acting in the reverse fashion, in that it is gaining potassium, possibly at the expense of sodium. This is the opposite of the situation in the distal tubule. Recent research has shown the complexity of electrolyte exchanges that are possible along the length of the nephron (Ussing *et al.*, 1960; and Windhager and Giebisch, 1965) and as both the nephron and the oviduct are essentially tubes of specialised epithelial cells it is interesting to consider the plumping process in the light of the kind of active ionic transport systems that have been demonstrated in the renal tubules.

The essential process in plumping is the large movement of water which must pass across the oviduct epithelium and then across the membranes of the egg. This could be accomplished by an initial large inward movement of sodium, about 1,500-2,000 micromoles which would build up the necessary osmotic gradient so that water would then move into the oviduct. If, at a subsequent stage in the shell gland, these extra sodium ions could be reabsorbed through what has now become a water-impermeable membrane system (*vide* the collecting tubules of the nephron) this could produce the necessary hypotonicity with reference to the white so that water could now move across the, as yet, uncalcified shell membrane. Clearly much light could be shed on this system by continuing to get samples at later stages in the shell

gland and by measurement of the potential fields across the wall of the oviduct in various situations and states of functioning (Windhager and Giebisch, 1965). Preliminary analysis of a few samples of shell gland plumping fluids have shown the expected sodium concentration at about the level found in blood plasma together with increased potassium concentration (15 mM). Hoover and Smith (1958), in their analysis of shell gland fluid also believed that some reabsorption of electrolytes must occur. This lends some support to the above considerations but so far no evidence has been obtained concerning the fascinating problem of how the added sodium is removed.

Another feature of interest is the way the yolk membrane after ovulation appears to be able to maintain concentration differences between the white and the yolk, particularly as these gradients change markedly. Smith, Wilson and Brown (1954) working with mature White Leghorns found the same general situation concerning sodium and potassium distribution between the yolk and the white i.e. white tends to be sodium-dominated and yolk to be potassium-dominated. However, the absolute quantities per equivalent egg are evidently rather higher than in our eggs. As far as electrolyte transport is concerned, this raises an interesting question concerning the changes that may have been produced in the efficiency of oviduct machinery, by the genetic procedures used in the development of the modern egg-producing bird.

The situation in the maturing follicle, where it would appear that some follicles produce yolk substance with relatively low concentrations of such important minerals as potassium and sodium, raises the issue of how important is a constancy of the yolk ionic milieu for the initial growth of the fertilised ovum. It would seem from the present results that there is no clear relationship between the transport of organic material to the yolk and the transport of key electrolytes. This poses questions about such matters as the mechanisms governing the quantities of minerals added to the accumulating yolk substance, and whether this machinery is linked in any way to the mechanism of ovulation.

Finally it is realised that the issues raised have wide implications concerning both fundamental biological machinery and such issues as egg size or egg hatchability. Many more questions are posed than can be answered but the purpose of the present paper will be amply served if further research can be stimulated into these important and fascinating aspects of follicular and oviducal functions.

#### *Acknowledgements*

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## RECOVERY OF YOLK PIGMENT IN THE LAYING FOWL

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### *Synopsis*

THE EXTENT to which  $\beta$ -carotene and the ethyl ester of  $\beta$ -apo-8'-carotenoic acid are deposited in egg yolks has been investigated using Light Sussex birds fed on a low pigment semi-synthetic basal diet. The criterion for deposition was based on the measurement of the amount of the pigment in the yolk rather than on an assessment of the total colour. The pigments were administered in either oil solution or as the crystalline materials in gelatine capsules, since this allowed the amount of pigment ingested to be assessed more accurately than if the pigments were dispersed in the feed. There was little evidence of deposition of the  $\beta$ -carotene but about 10 per cent of the ethyl ester of the carotenoic acid appeared in the yolks.

### *Introduction*

The majority of the colour in egg yolks is due to the presence of alcohol-soluble hydroxy derivatives of carotene, termed xanthophylls. The xanthophyll fraction was thought to be a homogenous pigment by Willstätter and Escher (1912), but it was later shown by Kuhn, Winterstein and Lederer (1931) to contain two compounds, zeaxanthin and lutein. The remaining 2-10 per cent of the colour was shown by Gillam and Heilbron (1935), and Grimbleby and Black (1952), to be due to the presence of  $\beta$ -carotene and cryptoxanthin. Fig. 1 shows the four pigments which are found in egg yolks under natural conditions:  $\beta$ -carotene, cryptoxanthin, zeaxanthin and lutein. These are all long-chain hydrocarbons with a  $\beta$ -ionone ring at each end of the molecule (Karrer and Jucker, 1950). The conjugated, unsaturated double-bond system is responsible for the colour.  $\beta$ -carotene is wholly hydrocarbon, while cryptoxanthin has one hydroxyl group on one of the rings. The two xanthophylls, lutein and zeaxanthin have hydroxyl groups in both rings and differ only in the configuration of the double-bonds in one of the rings. The data presented by Grimbleby and Black (1952) and by Kuhn *et al.* (1931) allow the relative distribution of the four pigments to be calculated and the results are shown in Table 1.

Reported values for pigment levels in foodstuffs vary widely, as the carotenoid content depends on the type and source of the ingredients, season, method of processing and time of storage. The highly unsaturated nature of the carotenoids renders them susceptible to oxidative destruction. This oxidation is accelerated by heavy metal salts, rancid fats, high temperatures and certain feedstuffs such as fishmeals (Rathmann and Daggy, 1961). Variations in analytical conditions make it

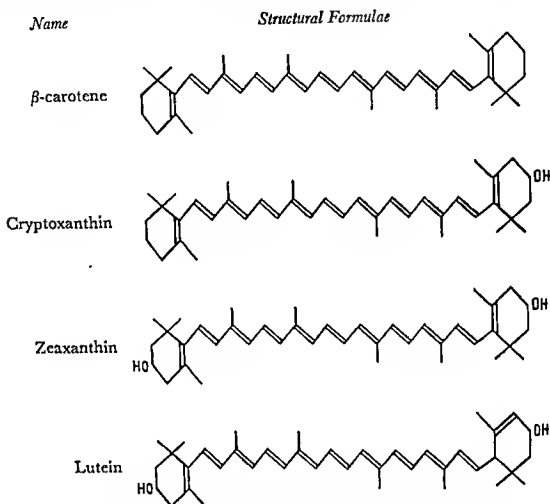


FIG. 1. Pigments appearing in egg-yolks under natural conditions.

difficult to find reproducible values for the pigment contents of feedstuffs. The common sources of pigments in British poultry feeds are maize, lucerne and grass-meal. An example of the variation in pigment content can be seen for the xanthophyll level in lucerne, where values as high as 960 mg./kg. and as low as 190 mg./kg. have been found (Goodwin, 1952; Ewing, 1963). It would appear that the xanthophyll fraction of lucerne is predominantly lutein (Bickoff, Livingston and Bailey and Thompson, 1954) and that of maize is zeaxanthin (Goodwin, 1952).

The difference in the relative concentrations of carotenes and xanthophylls in the egg and in the food is striking. In green foods the carotene:xanthophyll ratio is 1:3 whilst in eggs it can reach 1:30. Mattson and Deuel (1943) showed that this increase occurs mainly through the  $\beta$ -carotene and cryptoxanthin being metabolised to vitamin A rather than to any failure in absorption from the intestinal lumen.

There appear to be two major routes for the metabolism of  $\beta$ -carotene to vitamin A (Thommen, 1961; Brubacher, Gloor and Wiss, 1960). The  $\beta$ -carotene molecule may be split medially with the uptake of two molecules of water to yield two molecules of vitamin A. Alternatively, a gradual stepwise oxidation, starting from one end of the conjugated chain produces the apo-carotenes, the first being  $\beta$ -apo-8'-carotenal. Further oxidation yields  $\beta$ -apo-8'-carotenoic acid, which eventually oxidises to vitamin A. The vitamin A activity of the different carotenoids depends on whether the  $\beta$ -ionone ring structure is present once or twice in each molecule (Tiews, 1963). The  $\beta$ -ionone rings of the xanthophylls are modified by the presence of the hydroxy- or oxo-groups and are vitamin A inactive. Hughes and Payne (1937)

TABLE 1

*Distribution of pigments in egg yolks under natural conditions*

Pigment	Ratio*
$\beta$ -carotene	1
Cryptoxanthin	7.5
Zeaxanthin	75
Lutein	175

\*  $\beta$ -carotene taken as unity.

state that between 15 and 25 per cent of the total ingested carotenoids are usually deposited in the body tissues. The xanthophylls are laid down in the fat depots and shanks as esters and these are mobilised in the free state into the eggs during the laying period (Goodwin, 1952). Only about 8 per cent of the xanthophylls in eggs are in the esterified form (Peterson, Hughes and Payne, 1939). As early as 1919 Palmer and Kempster considered that xanthophylls are of no physiological importance and that mobilisation from the tissues to the eggs is merely due to the fact that the eggs are a convenient route for the excretion of fat-soluble compounds.

The present study has been undertaken to provide a foundation for future work which aims to determine the extent of the breakdown of the fat-soluble pigments to colourless products such as vitamin A. The factors in the diet which affect these processes will receive particular attention. All the breakdown products are not known and so the work is being limited to the estimation of particular compounds in the egg yolk. So far, studies have only been made with pure pigments rather than with ill-defined natural feeds which contain a wide variety of carotenoids. Much of the early work on avian carotenoid metabolism was done with pigmented feedstuffs or crude extracts, and although some work has been done with purified compounds the results have been expressed in terms of total colour or as consumer acceptability rather than as quantitative recoveries (Gillam and Heilbron, 1935; Peterson *et al.*, 1939; Ganguly, Mehl and Deuel, 1953; Marusich, Kadin and Bauernfeind, 1958; Marusich, De Ritter and Bauernfeind, 1960).

### *Materials and Methods*

An attempt has been made to establish a quantitative method for estimating the pigment content of egg yolks. The method used was based on one described by Goodwin (1955) for use with plant material.

The yolk and albumen are separated and the yolk is rolled on filter paper to remove adherent albumen. The lipids are then extracted three times with 80 ml. of acetone in a blender (Atomix 1 litre) under nitrogen. The combined filtrates are treated with 25 ml. of alcoholic potassium hydroxide for 15 min. under reflux and allowed to cool. The non-saponifiable material, including the carotenoids, is then separated from the saponified material by extraction with diethyl ether and water. Separation of the fatty acids in the aqueous phase at this stage prevents their interfering with the chromatographic separation of the pigments. The above saponification process converts the xanthophyll esters to free xanthophylls, which is desirable because the esters often exhibit the characteristics of carotenes on the chromatographic column.

The ether extracts are washed free from alkali with distilled water until the washings are neutral to phenolphthalein and then evaporated to dryness under reduced pressure in an atmosphere of nitrogen. The residual pigments are then dissolved in a minimum volume of petroleum ether (boiling range 40-60°C.) and the solution applied to a column of aluminium oxide 5 cm. long and 1 cm. in diameter, the activity of the aluminium oxide having been reduced by standing it overnight in methanol. It is found that the carotenes run through the column in the petroleum ether, while the xanthophylls are adsorbed in a band at the top; these can be eluted with 20 per cent ethanol in acetone. The various fractions are then diluted to a standard volume in petroleum ether and the characteristic absorption spectra plotted on a spectrophotometer (Unicam S.P. 700) between wave numbers 267 and 200  $\text{cm}^{-1}$ .

When the absorption spectrum of the first fraction was plotted, a large amount of material was present which distorted the absorption spectrum of the  $\beta$ -carotene to such an extent that when only a small quantity of  $\beta$ -carotene was present no precise estimation of the concentration could be made. The interfering material was not rendered water soluble on saponification; it absorbed light in the ultraviolet region of the spectrum and as it was precipitated by digitonin it was presumed to be a sterol. Many combinations of eluants and absorbents were tried in an effort to separate the material from the carotenes, without success. When the pigments were present in greater concentrations the effect of the interference was less marked and the following procedure was then devised to determine the carotene concentration.

An aliquot of the petroleum ether solution of the pigment is taken and the absorption spectrum of the total colour fraction plotted. The

remainder of the solution is then applied to the column, the xanthophylls separated from the other pigments and the absorption spectrum of the xanthophyll fraction plotted. The concentration of the carotene fraction is then calculated by difference.

Standard solutions of  $\beta$ -carotene and zeaxanthin were prepared and recovery experiments carried out. An egg yolk was divided into two equal portions and a known amount of pigment was added to one half. Both halves were subjected to the above analytical procedure and the recovery of the pigments was calculated by the difference between the values for the two halves. Table 2 shows the results.

TABLE 2

*Recoveries of pure compounds (the crystalline compound added to a halved egg yolk and subjected to the analytical procedure)*

Compound	Recovery per cent
$\beta$ -carotene	98.8
	99.8
	100.8
Zeaxanthin	99.0
	99.0
	98.8

Standard curves for the relationship between optical density and concentration have been prepared for  $\beta$ -carotene, zeaxanthin and the ethyl ester of  $\beta$ -apo-8'-carotenoic acid. The last is incorporated into gelatine beads at the level of 10 per cent and used in commercial laying rations as a pigment source under the trade name "Carophyll 10" (Roche Ltd). Hydrolysis of the ester during the saponification process gave recoveries of between 70 and 80 per cent for the "Carophyll 10". However, since the saponification is necessary to remove the large amounts of triglyceride fat present in the yolks and to prepare a solution of the pigments suitable for separation on the alumina columns, these low recovery values have been accepted.

To determine the amount of ingested pigment which appeared in the yolk, the birds were fed on the low pigment ration shown in Table 3. The ration was supplemented with vitamins and minerals, see Table 4, to raise the levels in the diet to the recommendations of the National Research Council (1960). Light Sussex hens from the Wye College flock in their first year of lay were used. To deplete the body stores of carotenoids 2 months on this ration were necessary. The ration maintained the birds in full lay, but a problem was encountered with the meal which because of its very fine powdery texture was unacceptable to the birds. Commercial feedstuffs manufacturers found it impossible to pellet the ration, but the problem was eventually solved by wetting the meal and pushing it through a coarse sieve to form crumbs which were dried at 100°C.

There are conflicting reports of the utilisation of  $\beta$ -carotene as a pigmenting agent. Kennedy (1935), Peterson *et al.* (1939) and Thumin (1962) report that  $\beta$ -carotene does not affect the colour of the yolk

TABLE 3  
*Low pigment ration*

Ingredients	per cent
Corn starch	61
Groundnut	20
Fishmeal (65 per cent protein)	6
Casein	3
Supplements (see Table 4)	10
	<hr/> 100 <hr/>
Crude protein (per cent $N \times 6.25$ )	15.7 per cent
Metabolisable energy	3,120 kcal./kg.

TABLE 4  
*Supplements for 1,000 lb. of complete ration*

<i>Vitamin Supplement</i>	
Vitamin A	2,500,000 i.u.
Vitamin D <sub>3</sub>	700,000 i.u.
Vitamin E	24,000 i.u.
Vitamin K	500.0 mg.
Choline	200.0 g.
Pantothenic acid	2.0 g.
Riboflavin	1.0 g.
Nicotinic acid	15.0 g.
Folic acid	100.0 mg.
Vitamin B <sub>12</sub>	1.0 mg.
Thiamine	1.0 g.
Pyridoxine	1.3 g.
Biotin	10.0 mg.
<i>Mineral Supplement</i>	
CaHPO <sub>4</sub>	10.0 kg.
CaCO <sub>3</sub>	15.2 kg.
NaCl	2.52 kg.
MgSO <sub>4</sub> ·7H <sub>2</sub> O	224.0 g.
MnSO <sub>4</sub> ·5H <sub>2</sub> O	8.8 g.
FeSO <sub>4</sub> ·7H <sub>2</sub> O	4.4 g.
CuSO <sub>4</sub> ·5H <sub>2</sub> O	350.0 mg.
ZnCl <sub>2</sub>	2.2 g.

to any measurable extent, whereas Pearson (1962) reports a lemon yellow colouration of yolks after incorporating  $\beta$ -carotene in the feed. To ascertain what happened when  $\beta$ -carotene was fed under our experimental conditions, the following experiments were conducted.

Food wastage makes it impossible to record accurately the amount of a food ingredient ingested, and so the pigments under investigation

were force-fed in single daily doses instead of being incorporated at low levels in the feed. This technique involved handling the birds daily, but this produced no ill effects if the birds were handled after 4.00 p.m. as by this time oviposition and ovulation were complete.

### *Experiment 1*

The only way in which the small amount of  $\beta$ -carotene for one day's dose could be measured out was to make up a standard solution of the pigment in petroleum ether and measure an aliquot of this. Attempts to transfer the pigment to lard or onto a small portion of the ration proved unsatisfactory, but it could be transferred to ethyl oleate by removing the petroleum ether by evaporation under nitrogen. The ethyl oleate solution was administered with a syringe and crop tube. This procedure proved unsatisfactory during the course of the experiment since the ethyl oleate is sufficiently viscous for considerable quantities to adhere to the sides of the tube and syringe. This necessitated the use of a large amount of ethyl oleate to flush out the apparatus.

An egg was collected from each of four birds before the experiment started. Two received a daily dose of 1 mg. of  $\beta$ -carotene in ethyl oleate for 20 days and two served as controls receiving ethyl oleate alone. All the eggs laid during the dosing period and for the following 10 days were collected and analysed. There were indications in the case of the birds receiving the pigment that the  $\beta$ -carotene levels were higher than in the eggs from the controls.

### *Experiment 2*

Bunnell, Marusich and Bauernfeind (1962) showed that dry preparations are better utilised than oil solutions as measured by their effect on egg yolk colouration so it was decided to feed  $\beta$ -carotene in the dry state in this experiment. The commercial pigment "Carophyll 10", which is fed as a dry preparation, was included for comparison. Two of the birds were to receive 20 doses of 1 mg. of  $\beta$ -carotene per day and so 20 mg. were weighed out and diluted with 180 mg. of starch. This enabled the daily 1 mg. to be weighed out in 10 mg. aliquots which were administered in gelatine capsules (Park, Davis & Co.). Since the "Carophyll 10" is already diluted in gelatine beadlets it could be weighed out directly into the capsules. Until use, the gelatine capsules were stored under nitrogen in stoppered tubes in a refrigerator.

### *Results*

The pigment contents of the eggs laid during Experiment 1 are shown in Table 5. One of the control birds stopped laying on day 8 and the other bird laid two soft shelled eggs which were lost. The treated birds showed a slight increase in the pigment content of the eggs on administration of  $\beta$ -carotene. However, none of the eggs laid in this experiment by the treated birds exhibited any visible colour



difference from those laid by the controls. Table 6 shows the  $\beta$ -carotene balance. The values for the pigment contents of yolks from the control birds were used to correct the pigment contents of the yolks from the treated birds for the pigment contributed by the basal ration. The values show the very low recovery for the administered  $\beta$ -carotene.

TABLE 5

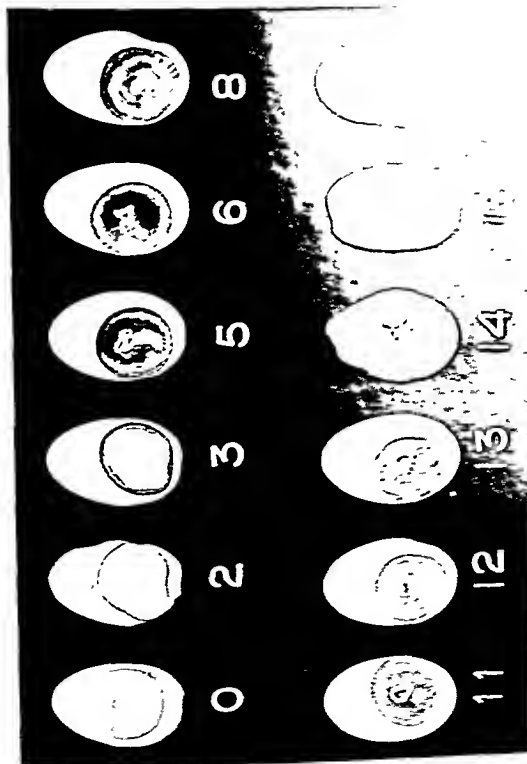
*$\beta$ -carotene recovered from eggs laid in Experiment 1 expressed as  $\mu\text{g. per egg yolk}$*

$\beta$ -carotene in ethyl oleate					
Day	Treated birds Replicates		Control birds Replicates		
	A	B	A	B	
0	3	5	>1	3	
1		3		2	
2	2	7	>1		
3				2	
4		7		3	
5		7			
6	3	5		9	
7					
8		6		4	
9		6			
10	19	5			
11	6	4	2		
12					
13	4	3	2		
14	4	4	2		
15	3	5			
16			2		
17	3	4	2		
18	3	5			
19		3			
20	2				
21	3	5	s.s.		
22		3	2		
23	4	3			
24	3	2			
25	2				
26		3			
27	2	3			
28	3	3			
29		3	s.s.		
30		3	2		

s.s. refers to soft shelled egg which was lost.

From \* to \*\* refers to dosing period.

Table 7 shows the values for the eggs laid in Experiment 2. Those from the control birds had a constant pigment level, but there were no indications of any increase due to the administered  $\beta$ -carotene. In marked contrast were the values for the yolks produced by the bird which received the "Carophyll 10", the yolks with more than 100  $\mu\text{g.}$



Longitudinal sections of cere glanthead buds from 1914  
study class of Caryophyllaceae

The numbers show the sequence in which the sections were taken from the first class

of pigment having a visibly acceptable degree of colour. Unfortunately values for only one bird are available.

The calculations of the recovery of the two pigments in this experiment are shown in Table 8. The results for the birds receiving the crystalline  $\beta$ -carotene indicate a negative recovery in the yolks. The corresponding values for "Carophyll 10" are also shown. The extinction coefficients of  $\beta$ -carotene and "Carophyll 10" are sufficiently similar to allow the values for the controls to be used directly to correct the pigment content of the "Carophyll 10" yolks. Since losses of "Carophyll 10" occurred in the analysis, these values should be regarded as minima.

TABLE 6

*$\beta$ -carotene balances for Experiment 1 expressed as  $\mu\text{g. pigment}$*

	Treated Replicates		Controls Replicates	
	A	B	A	B
Total pigment in yolks	70	105	14	14
Number of eggs	17	25	9	6
Average pigment per yolk			1.6	2.3
Pigment contribution of basal diet			2.0	
Pigment from basal diet in treated yolks	34	50		
Administered pigment recovered mean value		45		
20 mg. administered $\therefore$ recovery		0.2 per cent		

Plate 12 shows yolks of eggs laid by a bird which had received the daily dose of "Carophyll 10". The marked banding shows that the deposition of each dose of pigment must have occurred in less than 24 hours since each layer of pigmented yolk is separated from the next by a band of pigment-free material.

### *Discussion and Conclusions*

The present results confirm those of Kennedy (1935) and Thumin (1962), who could detect no colour change in egg yolks as a result of feeding  $\beta$ -carotene. Although in Experiment 1 a minute amount of  $\beta$ -carotene was shown to be deposited it was too small to be detected by any means other than chemical analysis. This suggests that the conversion of  $\beta$ -carotene to vitamin A is very rapid and corroborates the findings of Mattson and Deuel (1943). These workers accounted for the accumulation in eggs of xanthophylls rather than carotenes by the rapid metabolism of carotenes to vitamin A.

The results of Bunnell *et al.* (1962) showed that dry preparations of  $\beta$ -apo-8'-carotenal were deposited in yolks to a greater extent than when fed in cottonseed oil; however, our results obtained with  $\beta$ -carotene were contrary to this, perhaps because of a stabilising effect of ethyl oleate protecting the  $\beta$ -carotene from destruction in the intestinal tract.

Furthermore, Russell, Taylor, Walker and Polskin (1942) have suggested that carotene is better absorbed by adult hens from an oily medium.

TABLE 7

*$\beta$ -carotene and "Carophyll 10" recovered from eggs laid in Experiment 2, expressed as  $\mu\text{g. per egg yolk.}$*

Day	Controls Replicates		Crystalline $\beta$ -carotene Replicates		"Carophyll 10"
	A	B	A	B	
0		4	3	5	4
* <hr/>					
1					
2					40
3		>1	2	3	65
4		3	2	2	69
5				3	
6		3		2	68
7	8				86
8	5	3	3	2	112
9			2		164
10	3			3	
11		3			161
12	3	4	2	3	168
13		4		3	133
14	3		3		
15	4	3	3	2	152
16				2	190
17	1	3	3	2	145
18					133
19	1	4	2	2	
20			2	3	129
** <hr/>					
21		3			125
22	2			2	128
23		6		3	
24	2		2	3	92
25		2			107
26	2	3		3	
27	3				37
28		3			
29		4			9
30	+	+	+	+	6
31					7
32					10
33					
34					5
35					3 <sup>+</sup>

From \* to \*\* refers to dosing period.

+ Collection of eggs ceased.

The work of Marusich *et al.* (1960) showed that the level of deposition may vary from 2.2 per cent to 26.0 per cent depending on the pigment administered. The results for "Carophyll 10" compare favourably with those obtained for  $\beta$ -apo-8'-carotenal, which is the aldehyde form corresponding to "Carophyll 10" (see Table 9).

It is interesting to note that a compound which is intermediate in the conversion of  $\beta$ -carotene to vitamin A is deposited in the yolk to a far greater extent than  $\beta$ -carotene itself. It may be that in any given time only a limited amount of  $\beta$ -carotene and intermediates can be

TABLE 8

$\beta$ -carotene and "Carophyll 10" balances for Experiment 2, expressed as  $\mu$ g. pigment

	$\beta$ -carotene Replicates		Controls Replicates		"Carophyll 10"
	A	B	A	B	
Total pigment in yolks	29	47	36	56	2,370
Number of eggs	12	18	12	17	27
Average pigment per yolk			3.0	3.3	
Pigment contribution of basal diet			3.1		84
Pigment from basal diet in treated yolks	37*	56*			2,286
Administered pigment recovered	—	—			9.8 per cent
23.2 mg. administered $\therefore$ recovery					

\* The calculated pigment contribution of the basal diet to the treated eggs exceeded the determined content, indicating a negative recovery.

involved in the process of oxidation. Thus any sudden increase in concentration of one intermediate will result in a proportion of that intermediate being subjected to another fate. In this case a proportion of the "Carophyll 10" is deposited in the yolk and is thus unavailable for vitamin A synthesis by the pathway proposed by Brubacher *et al.* (1960).

TABLE 9

Comparison of results with those of other workers

	Pigment	Recovery in yolk per cent
Experiment 1	$\beta$ -carotene	0.2
Experiment 2	$\beta$ -carotene	0.0
Peterson <i>et al.</i> (1939)	$\beta$ -carotene	0.0
Thumin (1962)	$\beta$ -carotene	0.0
Experiment 2	$\beta$ -apo-8'-carotenoic acid ethyl ester*	9.8
Marusich <i>et al.</i> (1960)	$\beta$ -apo-8'-carotenal	8.3

\* "Carophyll 10".

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SOME ASPECTS OF THE LIPID  
METABOLISM OF THE CHICK EMBRYO

R. C. NOBLE AND J. H. MOORE

*National Institute for Research in Dairying, Shinfield, Reading, Berks**Synopsis*

A STUDY has been made of the lipids present in the yolks of fertile unincubated eggs obtained from normal and vitamin B<sub>12</sub>-deficient hens and in the yolks, livers and extrahepatic tissues of normal and vitamin B<sub>12</sub>-deficient chick embryos during the last 8 days of incubation. Esterification of cholesterol, mainly with oleic acid, occurred in the yolk as embryonic development proceeded. There appeared to be a preferential absorption from the yolk of phosphatidyl ethanolamine relatively rich in docosahexaenoic acid. Docosahexaenoic acid occurred in surprisingly high concentrations in the liver triglycerides. Considerable amounts of cholesterol oleate accumulated in the normal embryonic liver. At each stage of incubation studied, the concentrations of cholesterol ester in the livers of the normal embryos were greater, and on days 15 and 17 the concentrations of triglyceride were considerably less than the corresponding concentrations in the livers of the deficient embryos. Vitamin B<sub>12</sub>-deficiency resulted in markedly higher concentrations of stearic and palmitic acids and markedly lower concentrations of arachidonic and docosahexaenoic acids in the liver phospholipids. A deficiency of vitamin B<sub>12</sub> did not appear to influence the composition of the lipids isolated from the yolks or extrahepatic tissues of the developing chick embryo.

*Introduction*

Ferguson, Rigden and Couch (1955) conducted a comparative histological study of the tissues of normal and vitamin B<sub>12</sub>-deficient chick embryos and reported the presence of larger and more numerous fat vacuoles in the hepatic cells of the deficient embryos. Ferguson *et al.* (1955) concluded that a deficiency of vitamin B<sub>12</sub> resulted in some basic disturbance of the lipid metabolism of the chick embryo.

Extensive deposition of fat in the livers of vitamin B<sub>12</sub>-deficient rats has been observed by Bennett, Joralemon and Halpern (1951) and Hartmann (1959). However, Bennett *et al.* (1951) found that fatty

livers did not develop in rats given a vitamin B<sub>12</sub>-deficient diet supplemented with methionine. Thus there would appear to be a fairly logical explanation for the occurrence of fatty liver in the vitamin B<sub>12</sub>-deficient rat. It seems likely that the last step in the synthesis of the active methyl group of methionine from more highly oxidised one-carbon-atom compounds, i.e. the reaction between S-methyl-tetrahydrofolate and homocysteine, is catalysed in the liver by an enzyme system containing vitamin B<sub>12</sub> as a prosthetic group (Buchanan, Elford, Loughlin, McDougall and Rosenthal, 1964). At present the only known mechanism by which ethanolamine is methylated to produce choline involves a transmethylation reaction with S-adenosyl-methionine as the methyl group donor (Cantoni, 1951, 1953; Pilgeram, Hamilton and Greenberg, 1957). In the liver of the rat it would seem reasonable to envisage a reduced synthesis of choline from ethanolamine and hence a reduced synthesis of the main liver phospholipid, i.e. lecithin. In fact, Hartmann (1959) has reported reduced levels of lecithin in the livers of vitamin B<sub>12</sub>-deficient rats.

It has, of course, been well established that an extensive deposition of fat (Deuel and Hallmann, 1941) and a reduced synthesis of phospholipid (Perlman and Chaikoff, 1939; Perlman, Stillman and Chaikoff, 1940; Horning and Eckstein, 1946; Cornatzer and Cayer, 1950) occur in the liver of rats given diets deficient in choline. As might be expected rats given choline-deficient diets supplemented with lecithin do not develop fatty livers (Hershey and Soskin, 1931).

It seemed doubtful whether the occurrence of fatty livers in the vitamin B<sub>12</sub>-deficient chick embryo could be explained in terms of a reduced synthesis of choline by the embryo since the yolk of the hen's egg is an extremely rich source of lecithin (i.e. about 1.5 g. lecithin per egg). The question of accounting for the appearance of fatty livers in the vitamin B<sub>12</sub>-deficient embryo thus presents an interesting problem that has stimulated a general study in the authors' laboratory of the lipid metabolism of the normal and vitamin B<sub>12</sub>-deficient chick embryo. Some of the results of this study are now briefly described.

### *Materials and Methods*

Normal embryos were obtained from the eggs of Light Sussex hens that had been mated with Rhode Island Red cocks. These hens were kept on deep litter and were given a normal diet compounded for breeding birds.

Vitamin B<sub>12</sub>-deficient embryos were also obtained from the eggs of Light Sussex hens that had been given the all-vegetable protein diet of Coates, Harrison and Kon (1951). These hens were kept in batteries and were artificially inseminated twice weekly with semen from Rhode Island Red cocks. The hens were depleted of vitamin B<sub>12</sub> for periods varying from 6 to 15 months, by which time the hatchability of the



fertile eggs had fallen to 30 per cent or less. Control embryos were obtained from the eggs of Light Sussex hens that had been given for corresponding periods the same diet supplemented with 27.5  $\mu$ g. of vitamin B<sub>12</sub> per kg.

Embryos were removed from the eggs after various stages of incubation and the livers, extrahepatic tissues and yolk sacs were retained for lipid analysis by the techniques fully described by Moore and Doran (1961, 1962) and Noble and Moore (1964, 1965).

### Results

The results of the various experiments have been given in detail by Moore and Doran (1961, 1962) Noble and Moore (1964, 1965) and are, therefore, summarised only briefly in the present communication.

*The lipids of the yolks, livers and extrahepatic tissues of embryos derived from hens given a normal diet*

Fig. 1 shows the weights (g. per yolk) of the various lipid fractions in the yolks at various stages in the incubation process. During the first 13 days of embryonic development about 350 mg. of total lipid was absorbed from the yolk. From day 13 to day 15 a further 230 mg. of

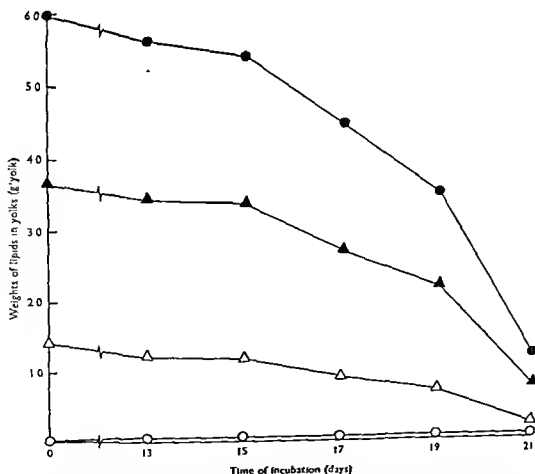


FIG. 1. Mean weights of total lipid (●), triglyceride (▲), phospholipid (△) and cholesterol ester (○) in the yolks at various stages of embryonic development.

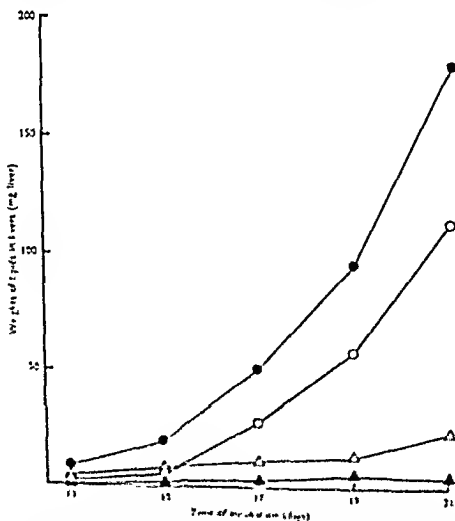
lipid was transferred from the yolk, but between day 15 and day 21 the absorption of lipid from the yolk sac increased to the extent that during the last 2 days of incubation, lipid was absorbed at the rate of over 1 g. per day. The concentration of triglyceride and phospholipid

TABLE 1

*Esterified cholesterol expressed as a percentage of total cholesterol in the yolk at various stages during incubation*

Time of incubation (days)		Time of incubation (days)	
0	4.2	17	28.0
6	4.0	18	28.7
9	4.1	19	37.0
12	11.1	21	39.0
15	17.0		

expressed as percentages of the total yolk lipid did not vary to any extent during incubation. It would appear that cholesterol is actively esterified in the yolk during incubation. This is clearly shown by the combined results of Tsuji, Brin and Williams (1955) and of Moore and Doran (1962) presented in Table 1. The weights (mg./liver) of the



2. Mean weights of total lipid (●), triglyceride (▲), phospholipid and cholesterol ester (○) in the liver at various stages of embryonic development.

various lipid fractions in the livers of the embryo during the last 9 days of incubation are shown in Fig. 2. After day 15 the pronounced increase in total liver lipid was accounted for mainly by the concomitant increase in cholesterol ester. Only relatively small increases in the amounts of phospholipid and triglyceride occurred from day 15 to day 21. The concentrations of the various lipid fractions in the dry matter of the liver of the developing chick embryo are shown in Table 2. The weights (g. per embryo) of the various lipid fractions in the extrahepatic tissues of the embryo during the last week or so of incubation are shown in Fig. 3.

TABLE 2

*Lipid composition (g./100 g. dry tissue) of the livers of normal chick embryos (mean values)*

Time of incubation (days)	15	17	19
Total lipid	25.7	29.0	45.1
Triglyceride	5.09	4.55	3.40
Sterol ester	8.71	13.33	31.5
Free sterol	1.19	1.22	1.15
Unesterified fatty acids	1.54	0.81	0.63
Phospholipid	8.64	9.03	7.03

As in the liver, the amount of total lipid increased as incubation proceeded. However, the concentration of lipid in the dry matter of the extrahepatic tissues (30 per cent on day 21) was considerably less than that in the dry matter of the liver (51 per cent on day 21). Unlike the changes in liver lipid that occurred during incubation, the increases in extrahepatic lipid were mainly due to triglyceride which accounted for as much as 75 per cent of the total extrahepatic lipid on day 21.

The fatty acid compositions of the cholesterol ester fractions isolated from the yolk, liver and extrahepatic tissues of the developing chick embryo are shown in Table 3. At all stages of incubation oleic acid was the main acid esterified with cholesterol in the yolk and the concentration of this acid in the cholesterol ester fraction increased progressively throughout the whole period of incubation. In spite of the large increases in the amounts of esterified cholesterol that occurred in the liver during embryonic development, the fatty acid composition of the cholesterol esters remained fairly constant. Cholesterol oleate accounted for almost 80 per cent of the total liver cholesterol esters. It is interesting to note that the fatty acid composition of the yolk cholesterol esters and the cholesterol esters of the whole embryo (liver plus extrahepatic tissues) was very similar at each stage of incubation (Table 4). The fatty acid composition of the phospholipid fractions isolated from the yolks, livers and extrahepatic tissues of the developing chick embryos are shown in Table 5. Of the various fatty acids present in the yolk phospholipids only docosahexaenoic acid, a relatively

minor component, showed any consistent decrease in concentration as development proceeded. This progressive decrease in the concentration of docosahexaenoic acid in the yolk strongly suggests that the developing embryo preferentially utilises those yolk phospholipids that contained large proportions of this  $C_{22}$  polyunsaturated acid. At all

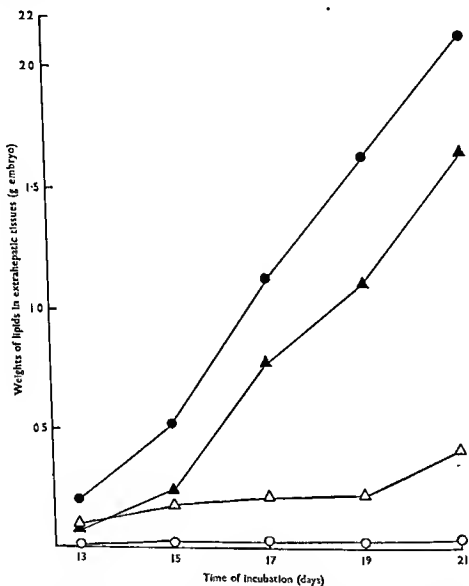


FIG. 3. Mean weights of total lipid (●), triglyceride (▲), phospholipid (Δ) and cholesterol ester (○) in the extrahepatic tissues at various stages of embryonic development.

stages of development, the fatty acid composition of the yolk phospholipids was characteristically different in certain respects from that of the liver phospholipids. The liver phospholipids contained considerably more stearic, arachidonic and docosahexaenoic acids and considerably less palmitic and oleic acids than did the yolk phospholipids. The concentrations of palmitic and stearic acid in the extrahepatic phospholipids were very similar to the concentrations of these two saturated acids in the yolk phospholipids. However, the extrahepatic phospholipids contained less oleic acid and more  $C_{20}$  and  $C_{22}$  polyunsaturated acids than did the yolk phospholipids.

The fatty acid composition of the triglyceride fractions isolated from

the yolks, livers and extrahepatic tissues of the developing chick embryo are shown in Table 6. With the exception of linoleic acid the fatty acid composition of the yolk triglycerides remained fairly constant during incubation. The concentration of docosahexaenoic acid in the liver triglycerides was surprisingly high and it seems unlikely that the docosahexaenoic acid in the liver triglycerides was derived from the

TABLE 3

*Fatty acid composition (molar percentage) of the cholesterol ester fractions obtained from the tissues of normal chick embryos*  
(mean values)

Time of incubation (days)	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
Yolk	0	15.0	25.9	39.7	3.8	1.3
	13	18.0	17.2	46.7	7.8	1.0
	19	6.6	14.4	67.6	4.3	0.7
Liver	13	3.5	9.8	75.7	3.1	1.4
	19	2.2	11.2	78.4	3.4	1.4
	19	13.5	17.7	42.8	5.3	5.0
Extra-hepatic tissues	13	13.5	17.7	42.8	5.3	5.0
	19	10.3	19.5	50.6	5.5	2.9

TABLE 4

*Comparison of the oleic acid concentrations in the cholesterol ester fractions of the yolk and whole embryo*

Time of incubation (days)	Yolk	Whole embryo
13	46.7	49.5
15	56.8	60.1
17	66.5	63.9
19	67.6	69.7
21	73.5	72.3

TABLE 5

*Fatty acid composition (molar percentage) of the phospholipid fractions obtained from the tissues of normal chick embryos*  
(mean values)

Time of incubation (days)	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
Yolk	0	29.5	14.5	25.4	15.9	4.4
	13	31.1	14.2	27.7	14.9	3.8
	19	29.8	13.3	29.3	17.7	3.8
Liver	13	22.7	11.6	8.2	23.9	20.4
	19	17.6	14.9	8.5	25.0	18.8
	19	17.6	14.9	8.5	25.0	18.8
Extra-hepatic tissues	13	34.1	9.0	20.9	16.5	7.8
	19	31.9	12.1	19.6	18.7	7.3

yolk triglycerides. However, it should be remembered that the docosa-hexaenoic acid content of the yolk phospholipids decreased markedly during incubation. In the triglycerides of both liver and extrahepatic tissues the proportion of arachidonic and docosa-hexaenoic acids decreased whereas the proportion of oleic acid increased as development of the embryo took place. With regard to the possibility of there

TABLE 6

*Fatty acid composition (molar percentage) of the triglyceride fractions obtained from the tissues of normal chick embryo*  
(mean values)

Time of incubation (days)		Fatty acids					
		16:0	18:2	18:1	18:0	20:4	22:6
Yolk	0	24.8	16.5	47.8	5.9	<0.2	<0.2
	13	25.6	12.0	49.9	6.3	0.3	0.5
	19	26.1	13.4	49.8	5.3	0.3	0.7
Liver	13	23.4	8.3	30.4	8.8	3.5	20.1
	19	21.6	7.6	37.5	9.5	2.8	15.5
Extra-hepatic tissues	13	29.8	13.8	34.7	7.8	2.3	6.4
	19	30.9	13.8	41.3	6.8	1.0	1.5

TABLE 7

*The fatty acid composition (molar percentage) of the various phospholipids present in the yolk of the unincubated egg*

	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
Polyglycero-phosphatide	31.3	5.0	18.4	15.8	2.0	<1.0
Phosphatidyl ethanolamine	21.2	9.0	15.0	29.5	12.9	8.0
Phosphatidyl serine	29.6	6.4	13.0	24.3	8.4	1.2
Phosphatidyl choline	35.5	14.0	26.5	14.7	4.3	2.7
Sphingomyelin	37.9	8.2	21.5	16.0	<1.0	<1.0
Lyso-phosphatidyl choline	28.0	15.0	18.2	22.6	<1.0	<1.0

being a preferential utilisation of a yolk phospholipid fraction particularly rich in docosa-hexaenoic acid, the results shown in Table 7 indicate that the major source of docosa-hexaenoic acid in the yolk phospholipids in the unincubated egg is in fact phosphatidyl ethanolamine. During the first fortnight or so of embryonic development there was a marked decrease in the concentration of phosphatidyl ethanolamine (expressed as a percentage of total phospholipid) in the yolk (Table 8). However, the concentration of docosa-hexaenoic acid in the yolk phosphatidyl ethanolamine also decreased during incubation

(Table 9). Thus, not only did there appear to be a preferential utilisation of yolk phosphatidyl ethanolamine during incubation but also a preferential utilisation of those phosphatidyl ethanolamine molecules containing docosahexaenoic acid.

*The effect of vitamin B<sub>12</sub> deficiency on the lipids of the yolks, livers and extra-hepatic tissues of the developing chick embryo*

The concentrations (g./100 g. dry tissue) of the various lipid fractions in the livers of the control and vitamin B<sub>12</sub>-deficient embryos (parent hens depleted of vitamin B<sub>12</sub> for 8 months) after 17 days of

TABLE 8

*The concentrations of the individual phospholipids (expressed as a percentage of total phospholipid) in the yolk during incubation*  
(mean values)

Time of incubation (days)	0	13
Polyglycerophosphatide	3.6	1.1
Phosphatidyl ethanolamine	16.0	9.2
Phosphatidyl serine	1.4	2.1
Phosphatidyl choline	69.5	79.7
Sphingomyelin	6.0	4.2
Lysophosphatidyl choline	3.6	3.8

TABLE 9

*The fatty acid composition (molar percentage) of the phosphatidyl ethanolamine present in the yolk during incubation*  
(mean values)

Time of incubation (days)	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
0	21.2	9.0	15.0	29.5	12.9	8.0
13	22.3	9.0	15.4	26.0	12.0	4.0
19	22.4	10.1	19.3	27.6	11.5	1.9

TABLE 10

*Lipid composition (g./100 g. dry tissue) of the livers of control and vitamin B<sub>12</sub>-deficient chick embryos (17 days)*  
(mean values)

	Control	Deficient
Total lipid	29.2	33.6
Sterol ester	13.3	7.4*
Triglyceride	4.6	14.0*
Phospholipid	9.0	9.6

\* Significantly different ( $P < 0.01$ ) from that in the control embryo.

incubation are shown in Table 10. The corresponding results for embryos derived from hens that had been depleted of vitamin B<sub>12</sub> for 15 months are shown in Table 11. When the parent hens had been depleted of vitamin B<sub>12</sub> for only 8 months there was no significant difference in the total lipid content of control and deficient embryos. Nevertheless, a histological study of portions of the control and deficient livers revealed the same differences in size of the fat vacuoles in the liver cells as had been noted by Ferguson *et al.* (1955). The results

TABLE 11

*Lipid composition (g./100 g. dry tissue) of the livers of control and vitamin B<sub>12</sub>-deficient chick embryos (17 days)*  
(mean values)

	Control	Deficient
Total lipid	27.3	37.9*
Sterol ester	9.7	2.5*
Triglyceride	2.9	17.0*
Phospholipid	10.3	8.5

\* Significantly different ( $P < 0.01$ ) from that in the control embryos.

TABLE 12

*Lipid composition (g./100 g. dry tissue) of the livers of control and vitamin B<sub>12</sub>-deficient chick embryos*  
(mean values)

Time of incubation (days)	Control		Deficient	
	15	19	15	19
Total lipid	25.7	45.1	28.1	37.5
Sterol ester	8.7	31.5	5.5*	22.4*
Triglyceride	5.1	3.4	8.2*	5.0*
Phospholipid	8.6	7.0	11.4	7.2

\* Significantly different ( $P < 0.01$ ) from that in the control embryos.

given in Table 11 show that there was a significantly higher concentration of total lipid in the liver of the deficient embryos derived from parent hens that had been deprived of vitamin B<sub>12</sub> for 15 months. However, as is evident from both Tables 10 and 11 the most striking effect of vitamin B<sub>12</sub> deficiency on the liver lipids of the chick embryo is observed on the proportions of the sterol ester and triglyceride fractions. Vitamin B<sub>12</sub> deficiency markedly reduced the concentration of sterol ester and increased the proportion of triglyceride. This effect is also apparent in the liver of 15- and 19-day embryos (Table 12) but the differences are not as striking as in the 17-day embryos. That the effects of vitamin B<sub>12</sub> deficiency on the liver lipids were apparently greater on day 17 than on day 19 may be explained by the fact that maximum mortality due to vitamin B<sub>12</sub> deficiency occurs at 17 days of incubation



and embryos surviving beyond this stage have greater reserves of the vitamin.

Vitamin B<sub>12</sub> deficiency did not appear to affect the concentration of total phospholipid in the embryonic livers but analysis of the individual liver phospholipids (by chromatography on columns of silicic acid) did reveal a slightly lower proportion of lecithin and a somewhat higher proportion of sphingomyelin in the phospholipids of the deficient

TABLE 13

*Phospholipid composition (percentage of total phospholipid) of the livers of control and vitamin B<sub>12</sub>-deficient chick embryos (17 days)*  
(mean values)

	Polyglycero- phosphatide	Kephalin	Phospho- inositide	Lecithin	Sphingo- myelin
+B <sub>12</sub>	8.7	23.8	5.4	54.5	6.9
-B <sub>12</sub>	9.1	23.8	6.7	51.3*	8.9*

\* Significantly different ( $P < 0.01$ ) from that in the control embryos.

TABLE 14

*Fatty acid composition (molar percentage) of the cholesterol ester fractions obtained from the livers of control and vitamin B<sub>12</sub>-deficient chick embryos*  
(mean values)

Time of incubation	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
+B <sub>12</sub> {						
13	2.3	12.7	77.2	2.0	2.2	1.9
17	2.4	13.7	77.3	2.8	1.6	0.9
19	2.6	12.4	77.1	3.3	2.1	1.2
-B <sub>12</sub> {						
13	3.9	9.1*	76.5	2.8	1.9	1.5
17	2.4	10.8*	79.9	2.6	2.1	0.9
19	2.2	10.7	78.4	2.4	3.6	1.0

\* Significantly different ( $P < 0.01$ ) from that in the control embryos.

embryonic livers (Table 13). The fatty acid compositions of the cholesterol ester fractions obtained from the livers of the control and vitamin B<sub>12</sub>-deficient embryos are given in Table 14. In spite of the pronounced effect of vitamin B<sub>12</sub> deficiency on the cholesterol ester content of the embryonic livers it should be noted that a deficiency of the vitamin did not appear to influence to any great extent the composition of the fatty acids esterified with cholesterol.

The fatty acid compositions of the triglyceride fractions obtained from the livers of the control and vitamin B<sub>12</sub>-deficient embryos are given in Table 15. On days 17 and 19 the concentration of palmitic, and on days 13 and 17 the concentration of linoleic, acid in the liver triglycerides of the deficient embryos were significantly greater than the corresponding concentrations in the liver triglycerides of the control

embryos. On the other hand, vitamin B<sub>12</sub> deficiency reduced the concentration of oleic acid in the triglycerides of the embryonic liver. The fatty acid compositions of the phospholipid fractions obtained from the livers of the control and vitamin B<sub>12</sub>-deficient embryos are shown in Table 16. Although the effect of vitamin B<sub>12</sub> deficiency on the levels of phospholipid in the embryonic liver was not very marked (Tables

TABLE 15

*Fatty acid composition (molar percentage) of the triglyceride fractions obtained from the livers of control and vitamin B<sub>12</sub>-deficient chick embryos (mean values)*

Time of incubation (days)	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
+B <sub>12</sub> { 13	23.3	12.5	33.2	9.4	5.0	11.7
17	22.1	12.5	37.9	9.6	4.0	9.2
19	21.1	13.6	39.1	10.1	4.3	8.1
-B <sub>12</sub> { 13	29.2	15.8*	26.2*	8.3	4.7	11.2
17	33.0*	16.6*	24.7*	8.2	4.2	7.6
19	30.8*	15.5	27.1*	9.7	3.9	7.5

\* Significantly different ( $P < 0.01$ ) from that with normal embryos.

TABLE 16

*Fatty acid composition (molar percentage) of the phospholipid fractions obtained from the livers of control and vitamin B<sub>12</sub>-deficient chick embryos (mean values)*

Time of incubation (days)	Fatty acids					
	16:0	18:2	18:1	18:0	20:4	22:6
+B <sub>12</sub> { 13	22.4	9.9	7.3	24.6	26.2	6.5
17	18.4	15.0	7.9	26.9	22.1	7.1
19	16.9	14.1	7.7	27.5	22.3	8.2
-B <sub>12</sub> { 13	28.8*	11.0	9.6	32.5*	12.6*	2.3*
17	24.4*	13.8	9.4	33.8*	12.4*	3.0*
19	25.3*	13.2	8.3	34.4*	11.2*	4.2*

\* Significantly different ( $P < 0.01$ ) from that in the normal embryos.

TABLE 17

*Unsaturated:saturated fatty acid ratios in the phospholipid fractions obtained from the livers of control and vitamin B<sub>12</sub>-deficient chick embryos (mean values)*

Time of incubation (days)	Control	Deficient
13	1.06	0.58
15	1.17	0.64
17	1.15	0.67
19	1.18	0.62

10, 11) the effect on the concentrations of certain of the fatty acids in the liver phospholipids was particularly striking. In the phospholipids of the deficient embryonic livers the concentrations of stearic and palmitic acids were consistently greater and the concentrations of arachidonic and docosahexaenoic acids consistently less than the corresponding concentrations of these acids in the phospholipids of the control embryonic livers. Attention should be drawn to the fact that the unsaturated:saturated fatty acid ratio in the phospholipids of the deficient embryonic livers were little more than half the corresponding ratios in the phospholipids of the control embryonic liver (Table 17). Vitamin B<sub>12</sub> deficiency appeared to have no effect whatsoever on the concentration of the major lipid fractions in the extrahepatic tissues or in the yolks at the various stages of the incubation process. The fatty acid composition of the major lipid fractions of the yolk were not affected by vitamin B<sub>12</sub> deficiency.

### *Discussion*

The detailed mechanism by which the yolk lipids are taken up by the yolk sac membrane is as yet unknown. The yolk sac is particularly well vascularised but contains no lymphatic system (Romanoff, 1960). The yolk lipids must therefore be absorbed by the epithelial cells of the yolk sac and transported to the embryo by means of the blood system. It is not clear to what extent the yolk lipids are enzymically degraded before absorption, for although lipase and "lecithase" activities have been demonstrated in the yolk as early as the fourth day of incubation (Needham, 1931), the initially low concentrations of monoglycerides, diglycerides, free fatty acids and lysophosphatides in the yolk of the early embryo do not appear to increase as development proceeds. (Moore and Doran, 1962; Noble and Moore, unpublished observations). At present it is difficult to envisage a reason for the progressive esterification of cholesterol with oleic acid in the yolk during incubation, but the relatively high concentration of cholesterol ester in the lipid of the chylomicrons and lipoproteins isolated from the plasma of the chick embryo (Schjeide, 1963) suggest that the cholesterol ester synthesised in the yolk might play some essential role in the assembly of a particular lipoprotein complex, in which form the lipid is transported from the yolk to the embryo.

Isaacs, Davies, Ferguson, Reiser and Couch (1964) compared the triglyceride and phospholipid fatty acids in the yolk of the unincubated egg with those in the yolk after incubation for 20 days and concluded that during development the embryo preferentially utilised the C<sub>20</sub> polyunsaturated acids and the oleic acid present in the yolk triglycerides and the C<sub>18</sub>, C<sub>20</sub> and C<sub>22</sub> polyunsaturated acids of the yolk phospholipids. However, the results of our more detailed investigation show that only the docosahexaenoic acid present in the yolk phospholipids

is selectively utilised by the growing embryo. It seems that this preferential utilisation of the docosahexaenoic acid of the yolk phospholipids is, to some extent at least, a reflection of the preferential utilisation by the embryo of the yolk phosphatidyl ethanolamine. The question now arises whether the embryo has a specific requirement for phosphatidyl ethanolamine or for the docosahexaenoic acid that the phospholipid contains. If indeed the embryo has a particular requirement for docosahexaenoic acid it seems strange that this acid after its preferential absorption from the yolk should be utilised in substantial amounts for triglyceride synthesis in the liver. The different fatty acid patterns observed for the phospholipids of the yolk, liver and extra-hepatic tissues are consistent with the findings of Hevesy, Levi and Rebbe (1938) and Budowski, Bottino and Reiser (1961) that phospholipids transported from the yolk to the embryo are immediately degraded to products that are either utilised to resynthesise new phospholipids necessary for embryonic growth or oxidised to furnish energy. Although Budowski *et al.* (1961) maintain that the yolk triglyceride that is not oxidatively metabolised by the embryo is retained without degradation and resynthesis, the rather unusual fatty acid composition of the triglycerides of the embryonic liver must indicate that new triglyceride molecules were actively synthesised in the liver during incubation. The reason for the presence of such high levels of docosahexaenoic acid in the triglyceride of the 13-day embryo is an intriguing problem worthy of further investigation.

Until a solution is found to the problem of why such large quantities of cholesterol oleate accumulate in the liver of the normal chick embryo during development, there seems little prospect of explaining why this characteristic accumulation of cholesterol oleate is reduced so markedly by a deficiency of vitamin B<sub>12</sub>. Nevertheless, as pointed out by Moore and Doran (1961), the analogy between the difference in the liver lipids of the normal and vitamin B<sub>12</sub>-deficient chick embryo and the difference in the blood lipids of pernicious anaemia patients before and after treatment (Williams, Erickson, Bernstein, Hummel and Macy, 1937) is very striking and points to a general participation of vitamin B<sub>12</sub> in lipid metabolism. By far the most consistent effect of vitamin B<sub>12</sub> deficiency on the constituent fatty acids of the various liver lipids of the chick embryo was that observed on the fatty acids of the total phospholipid fraction. The effect of vitamin B<sub>12</sub> deficiency on the composition and structure of the individual phospholipids of the embryonic liver is now being studied in some detail.

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# SOME EFFECTS OF ADRENOCORTICOTROPIC HORMONE ON BURSECTOMISED AND INTACT CHICKENS

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## *Synopsis*

THE EFFECTS of bursectomy and ACTH upon the adrenal gland of the young fowl have been investigated.

Following an injection of ACTH it was found that both sham-operated and bursectomised birds exhibit a fall in the cholesterol content of the adrenal gland. The fall was caused by a decrease in the ester cholesterol.

Hyperglycaemia and glycogenesis could be demonstrated within 2 hr. of an intramuscular injection of ACTH. It was found that there was a significant difference in the response of bursectomised chicks after this time—whilst the blood sugar concentration of the sham-operated birds continued to rise there was no further elevation in the concentration of blood sugar of the bursectomised birds.

Although bursectomy led to a significant reduction in the ascorbic acid content of the adrenal gland of the 3-week-old bird, it was found that ascorbic acid metabolism was not affected. It is therefore concluded that the adrenal gland of the young fowl is able to synthesise corticosteroids without a depletion of its ascorbic acid.

## *Introduction*

The bursa of Fabricius, a lympho-epithelioid gland peculiar to immature birds, is situated on the dorsal wall of the proctodaeum. Its functions have not been completely elucidated, although its importance in conferring immunological competence upon the bird is established (Chang, Glick and Winter, 1955; Glick, Chang and Jaap, 1956; Mueller, Wolfe and Meyer, 1960; Glick, 1962). In addition, evidence has been presented that there is an interrelationship between the bursa and the metabolism of ascorbic acid in the adrenal gland (Perek and Eilat, 1960).

It is well known that a rapid depletion of ascorbic acid from the adrenal glands occurs when mammals are stressed with exogenous

adrenocorticotrophic hormone (ACTH) (Sayers, Sayers, Lewis and Long, 1944; Sayers, Sayers; Liang and Long, 1946; Long, 1947; Elton and Zarrow, 1955; Howard and Constable, 1958; Elton, Zarrow and Zarrow, 1959), but this response has not been observed in the young fowl (Jailer and Boas, 1950; Howard and Constable, 1958; Newcomer, 1959). However, Perek, Eckstein and Eshkol (1959), Perek and Eckstein (1959) and Perek and Eilat (1960) found that it was possible to elicit such a depletion in the adult fowl after an injection of ACTH, a finding contrary to that of Elton and Zarrow (1955) and Elton *et al.* (1959). Perek and Eilat (1960) were further able to show that this response was dependent upon there being no bursal tissue present. In bursectomised young chickens a single intravenous injection of ACTH brought about a significant fall in the ascorbic acid content of the adrenal glands within 1 hr. These findings of Perek and his co-workers, however, were not confirmed by the experiments carried out by Wolford and Ringer (1962), who used adult birds.

It has also been shown that in mammals ascorbic acid depletion is accompanied by a similar fall in the cholesterol content of the adrenal glands (Sayers, Sayers, Fry, White and Long, 1944; Sayers *et al.*, 1944, 1946; Long, 1947; Elton and Zarrow, 1955; Howard and Constable, 1958; Elton *et al.*, 1959). Similar results have been obtained with both the intact immature and mature fowl (Slover, 1955; Howard and Constable, 1958; Siegel, 1962). More recently Sato and Glick (1964) have shown that bursectomy does not affect this response.

There appears to be some confusion as to whether an injection of ACTH leads to hyperglycaemia and glycogenesis in the starving fowl. Howard and Constable (1958), Siegel and Beane (1961), Bell (1961) and Siegel (1962) have all demonstrated the hyperglycaemic response whilst Howard and Constable (1958) and Brown, Brown and Meyer (1958) have demonstrated glycogenesis. Urist and Deutsch (1960), however, were unable to show the development of a hyperglycaemic state. This finding throws some doubt upon the efficacy of the ACTH itself. Elton (1957) has stated that bovine ACTH is not active in the bird. Urist and Deutsch (1960) used a freeze-dried preparation. Such a preparation was shown by Bell (1961) to be inactive but that the ACTH prepared by Armour Laboratories was active in the bird.

The experiments reported here were designed to attempt to determine whether the bursa of Fabricius is concerned in ascorbic acid metabolism in the adrenal gland and whether it is involved in any way with the functioning of the gland.

#### *Materials and Methods*

*Fowls.* All experiments were carried out on 3-week-old Brown Leghorn chickens of both sexes. Bursectomy or sham-bursectomy was carried out at 1 day of age. The technique was essentially that of



Mueller *et al.* (1960). Following the operations, the birds were reared normally on a standard chick starter ration (for the formula see Freeman, 1963). Before the experiments were begun the birds were starved overnight. Water was available *ad libitum*.

*Preparation and Administration of ACTH.* Fast-acting, lyophilised ACTH (Acthar, Armour) was dissolved in physiological saline to give a concentration of 50 i.u./ml. Injections were made into the thigh muscles (5 or 10 i.u./bird).

*Analytical Methods.* After a sample of blood has been taken by frontal heart puncture the bird was killed by cervical dislocation. Coagulation and glycolysis were prevented by adding potassium oxalate and sodium fluoride at a concentration of 3 mg. and 1 mg./ml. whole blood.

A sample of liver was removed immediately after killing the bird and dropped into a weighed tube containing 5 ml. 30 per cent (w/v) potassium hydroxide. The adrenals were dissected out, blotted, weighed and homogenised in 7 ml. 6 per cent (w/v) trichloroacetic acid.

Blood sugar was estimated by the method of Haslewood and Strookman (1939). Liver glycogen was precipitated according to the method of Good, Kramer and Somogyi (1933) and after hydrolysis the concentration of the glucose was determined.

Ascorbic acid was determined by the technique of Roe and Kuether (1943).

Cholesterol was extracted from adrenal tissue which was stored at  $-10^{\circ}\text{C}$ . by the method of Svatek and Knobloch (1962), and after separation into its free and ester forms by chromatography on activated alumina (see Varley, 1962) the concentrations of cholesterol were determined by the method of Zlatkis, Zak and Boyle (1953).

*Statistical Analysis.* A three-way analysis of variance was carried out, the factors being group, bursectomy and time.

*Experimental Design.* In preliminary experiments it was found that the bursa of Fabricius reached its greatest relative weight (mg./100 g. body weight) at 3 weeks of age—see Fig. 1. Accordingly all the experiments were carried out at this age, a time at which the influence of the bursa might be expected to be at its greatest.

*Presentation of Results.* Following the injection of ACTH it was noted that the weight of the adrenal glands was affected in a most inconsistent manner; not only was there a variable response between groups but also within groups (Table 2). No explanation can be offered, but in view of this finding all the concentrations of ascorbic acid and cholesterol have been given in absolute terms per pairs of adrenals.

Four separate experiments were carried out, two using 5 i.u. ACTH/bird and two using 10 i.u./bird. No statistical difference was noted between these results. Consequently they have all been considered together.

### Results

#### *Effects of Bursectomy upon the Fowl*

It will be seen from Table 1 that bursectomy did not have any significant effect upon the body weight, adrenal weight, total or ester cholesterol concentration in the adrenal glands, or upon the blood sugar concentration, although the effect was not consistent from group to group. There was, however, a consistent and significant reduction in the ascorbic acid concentration of the adrenal glands of the bursectomised chickens ( $P < 0.001$ ).

#### *Effect of a Single Injection of ACTH upon Bursectomised and Sham-operated Chickens*

**Adrenal Weight.** The results were extremely variable (Table 2). In two of the four groups of bursectomised chickens there was a significant fall in adrenal weight, but only in group 1 sham-operated birds was there a similar change. It is particularly noteworthy, however,

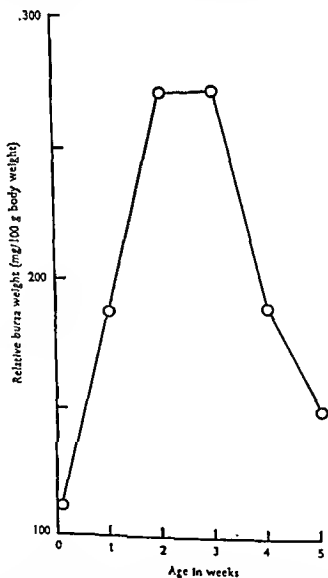


FIG. 1. The relative growth curve of the bursa of Fabricius of Brown Leghorn chickens during the first five weeks of life.

that there was no evidence of adrenal hypertrophy 6 hr. after the injection of ACTH although a transient rise of doubtful significance was noted 2 hr. after injection.

*Adrenal Ascorbic Acid.* Results were variable with both bursectomised and sham-operated birds. The only significant fall was noted with group 2 sham-operated birds. The bursectomised birds of group 3 showed a significant rise in adrenal ascorbic acid (Table 3).

*Adrenal Cholesterol.* Consistent results were obtained in all the

TABLE 1

*Effects of bursectomy upon the fowl*

	No. of obs.	Bursectomised	Sham-operated	Significance
Body weight (g.)	48	98.6 $\pm$ 4.3*	102.7 $\pm$ 4.3	Not significant
Adrenal weight (mg.)	48	18.9 $\pm$ 0.98	18.8 $\pm$ 0.98	Not significant
Total cholesterol ( $\mu$ g.)	21	323.4 $\pm$ 32.9	302.9 $\pm$ 32.9	Not significant
Ester cholesterol ( $\mu$ g.)	21	276.1 $\pm$ 30.7	239.8 $\pm$ 30.7	Not significant
Blood sugar (mg./100 ml.)	24	238.9 $\pm$ 11.5	235.1 $\pm$ 11.5	Not significant
Adrenal ascorbic acid ( $\mu$ g.)	24	28.5 $\pm$ 2.8	35.3 $\pm$ 2.8	P < 0.001

\* Mean  $\pm$  S.E.M.

TABLE 2

*Effects of ACTH upon the weight of the adrenal glands of bursectomised and sham-operated birds. Each figure is the mean of 48 observations*

Standard error of the mean = 0.98

Hours after injection	1		2		3		4	
	Burs.	Sham	Burs.	Sham	Burs.	Sham	Burs.	Sham
0	21.0	17.3	15.3	20.3	20.0	18.4	19.2	19.4
2	20.1	18.9	19.3*	21.5	24.9*	20.6	17.0	18.7
6	16.3*	11.6*	15.7	18.2	12.5*	20.2	17.3	19.5

\* Figure differs significantly from the initial weight.

TABLE 3

*Effects of ACTH upon the total content of ascorbic acid ( $\mu$ g.) of the adrenal gland of bursectomised and sham-operated birds. Each figure is the mean of 24 observations*

Standard error of the mean = 2.8

Hours after injection	1		2		3		4	
	Burs.	Sham	Burs.	Sham	Burs.	Sham	Burs.	Sham
0	32.1	36.0	24.1	34.7	26.3	30.4	31.6	40.0
2	32.0	28.0	25.0	27.5	28.0	25.6	30.7	37.5
6	28.9	37.0	30.4	24.3*	33.3*	25.7	31.0	37.2

\* Significantly different from initial concentration (P < 0.05).

experiments, thereby allowing amalgamation of the figures. The response was found to be similar in bursectomised and sham-operated birds. Within 2 hr. of the administration of ACTH there was a detectable fall in the total cholesterol content of the adrenals. This was particularly marked at 6 hr. ( $P < 0.001$ ). It will be noted from Fig. 2 that by the sixth hour there was a little evidence to suggest that the response was almost maximal for the bursectomised birds, whilst the sham-operated birds continued to show the same depletion rate as they had at 2 hr. It will be further noted that the depletion was caused mainly by a fall in the esterified cholesterol.

*Blood Plasma Sugar.* Again the results could be amalgamated. It will be seen from Fig. 3 that there were distinct rises of approximately the same magnitude within 2 hr. in both the bursectomised and the sham-operated birds. However, at 6 hr. post-injection the response was found to be significantly different. Whilst the blood sugar concentration continued to rise in the sham-operated birds to a level of 317 mg./100 ml., that of the bursectomised birds remained at a constant level of 279 mg./100 ml. This difference in response was found to be significant at the 0.1 per cent level.

*Liver Glycogen.* Only one experiment was carried out. Glycogenesis was demonstrated on both bursectomised and sham-operated birds. The rise in the liver glycogen was significant after 6 hr. in the sham-operated birds ( $P < 0.05$ ), but only approached significance in the bursectomised birds (Table 4).

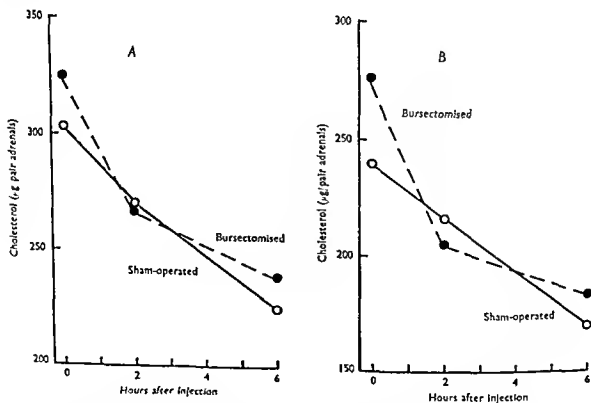
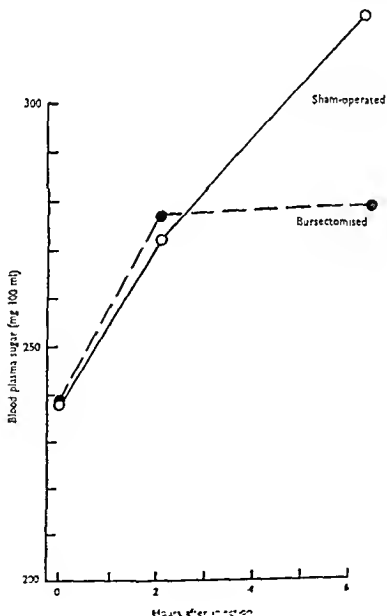


FIG. 2. The effect of ACTH upon the adrenal cholesterol of bursectomised and sham-operated birds. Each point is the mean of 21 observations. A. Total cholesterol S.E.M. = 32.9 µg. B. Ester cholesterol S.E.M. = 30.7 µg.

### Discussion

The adrenocorticotrophic hormone preparation used in these experiments (Acthar, Armour) was found to be active. Evidence of hyperglycaemia and adrenal cholesterol depletion was obtained within 2 hr. of an intramuscular injection of the hormone. The view of Elton (1957) that all preparations from bovine sources have no activity in the bird cannot therefore be accepted as correct.

Whilst there were very variable effects upon the adrenal glands after the administration of ACTH, there was no evidence of hypertrophy as Perek and Eilat (1960) were able to show. Rather the present results confirm the general impression that the adrenal gland of the fowl is relatively insensitive to stress when measured by the degree of hypertrophy. Conner (1959), Siegel and Beane (1961) and Wolford and Ringer (1962) were all unable to detect hypertrophy after a single dose



of ACTH, whilst Newcomer and Connally (1960) found that the adrenal hypertrophied only after 12 days' treatment with 6 i.u. ACTH/day.

It is generally accepted that there is no adrenal ascorbic acid depletion in the young fowl with an intact bursa (Jailer and Boas, 1950; Howard and Constable, 1958; Perek *et al.*, 1959; Perek and Eilat, 1960) and this has again been confirmed here. All the evidence that the bursa of Fabricius influences ascorbic acid metabolism in the adrenal gland is due to Perek and his co-workers (Perek and Eckstein, 1959; Perek and Eilat, 1960). In the present experiments it was found that although bursectomy led to a highly significant fall in the normal concentration of the vitamin in the gland (Table 1) there was no evidence that the absence of the bursa resulted in depletion when the

TABLE 4  
*Effect of 5 i.u. ACTH on the glycogen content of the liver*

Hours after injection	Bursectomised mg./g.	Shams mg./g.
0	0.5 ± 0.10 (5)	1.0 ± 0.30 (6)
2	1.0 ± 0.37 (5)	1.7 ± 0.33 (6)
6	3.8 ± 2.33 (3)	4.6 ± 1.30 (6)*

\* Significant rise between 0 and 6 hr.

bird was stressed with ACTH. The results from the bursectomised birds are consistent, rather with those of Elton *et al.* (1959) and Wolford and Ringer (1962) who found no ascorbic acid depletion in adult birds. It must be pointed out, however, that the present workers administered the ACTH by a different route and that a different breed of fowl was used.

It is perhaps noteworthy that other types of stress, such as heat and cold, have also been found not to influence the adrenal ascorbic level (Elton and Zarrow, 1955; Elton *et al.*, 1959) whilst chicks infected with *Eimeria tenella* show a significant elevation in adrenal ascorbic acid (Challey, 1960, 1962).

The finding that adrenal cholesterol is depleted following an injection of ACTH is in agreement with the work of Howard and Constable (1958), Siegel and Beane (1961), Siegel (1962) and Sato and Glick (1964), but is contrary to the findings of Elton and Zarrow (1955), Elton *et al.* (1959) and Newcomer (1959). The failure of these latter authors to detect any cholesterol depletion may have been due, in part, to either a non-active preparation of ACTH or that the correct dose/time relationship had not been evaluated. As would be expected the present experiments show that the fall in the total cholesterol concentration from the adrenal was due mainly to the utilisation of the esterified form.

The rise in the blood plasma sugar concentration and the deposition of glycogen in the liver of the starving bursectomised and sham-operated bird was noted within 2 hr. of the administration of ACTH thereby

demonstrating that the synthesis and the secretion of glucocorticoids takes place at this age.

It is of great interest that there should be such a marked difference in the blood sugar response of the bursectomised birds. Whilst a hyperglycaemic state was still developing after 6 hr. in the sham-operated birds, the maximal response of the bursectomised birds appears to have been reached between 2 and 6 hr. This apparent interference with carbohydrate metabolism has not been previously described and at the present state of knowledge no explanation can be advanced.

The results confirm those of Nagra, Baum and Meyer (1960) that the adrenal gland of the young chicken is able to actively synthesise and secrete corticosteroids and it appears that these processes do not involve the utilisation of ascorbic acid. Furthermore bursectomised birds cannot be considered to be of any greater value in assessing stress than the normal fowl.

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# THE EFFECTS OF GLUCAGON AND INSULIN ON THE PLASMA GLUCOSE AND UNESTERIFIED FATTY ACIDS OF THE DOMESTIC FOWL

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## *Synopsis*

THE LEVELS of plasma FFA in both laying and immature birds were not depleted by intravenous injections of glucose (1.5 g./kg. body weight) which doubled the level of plasma glucose for 15-30 min. Insulin (1.5 i.u./kg.) depleted plasma glucose in both starved and fed birds independent of the physiological state and induced an immediate and large increase in the plasma FFA. Glucagon also induced a large increase in plasma FFA simultaneously with the increase in plasma glucose, in both laying and immature birds, and independently of whether the birds were fasted or fed. These effects of insulin and glucagon were not abolished by pretreating the birds with reserpine (10 mg./kg. body weight) or with hexamethonium bromide (5 mg./kg. body weight). It is considered that the response in plasma FFA to glucagon may be a result of the direct action of this hormone on fatty acid release from the adipose tissue and that insulin promotes a similar response by stimulating an increased release of glucagon from the pancreas.

lipids increased markedly, shortly before laying commenced, the quantities of FFA reaching some  $4,000\mu$  equiv/litre of plasma before falling to lower levels when the first egg was laid (Heald and Badman, 1963). It was also demonstrated that the increased levels were not the result of impaired utilisation of fatty acids since isotopic clearance studies with palmitate  $C^{14}$  showed that the clearance was greatest when the fatty acid concentration was highest. Of several factors found to increase the levels of plasma FFA in the bird, oestrogens were highly effective (Heald and Rookledge, 1964). It was noted, however, that under such conditions no change occurred in the levels of plasma glucose. Further, an earlier study by Bell (1957) had shown that the levels of blood glucose were the same in both laying and immature birds.

In non-ruminant mammalian species, it appears to be well established that the levels of plasma FFA and glucose are determined partly by the caloric need of the animal and partly by the availability of glucose (Masoro, 1962). Thus low levels of plasma FFA are found in situations in which the level of plasma glucose has been raised by injection of glucose or glucagon, or the utilisation of glucose increased by administration of insulin. Conversely, high levels of plasma fatty acids are associated with situations such as starvation in which the availability of glucose is reduced. More recently, in the ruminant, principally the dairy cow, the responses of plasma FFA to feeding, fasting and to the administration of glucose and insulin have been shown to resemble those found in the non-ruminant mammal (Kronfeld, 1965).

The apparent independence of plasma FFA and glucose levels in the bird therefore prompted an examination of the effects of exogenous glucose, insulin and glucagon on the plasma FFA.

#### *Materials and Methods*

*Birds.* The birds used were a white Leghorn cross (CCA  $\times$  CRM) supplied by Cyril Thornber Ltd, Mytholmroyd, Halifax, Yorkshire. They were housed in individual cages in windowless rooms at an average temperature of  $12-8^{\circ}\text{C}$ . Laying birds were subjected to 14 hr. light in each 24 hr. commencing at 6.00 a.m. and ending at 8.00 p.m. Immature birds were similarly housed and subjected to 10 hr. light in each 24 hr. commencing at 8.00 a.m. and ending at 6.00 p.m. Food and water was supplied *ad libitum*.

*Blood Samples.* Samples of blood (1-1.5 ml.) were withdrawn via the wing veins into a syringe wetted with aqueous heparin (750 i.u./ml.) and transferred to tubes containing 10 mg. of the oxalate mixture of Wintrobe (1942). Plasma was obtained by centrifuging within 20 min. of the blood being collected. The total number of samples taken from any one bird was restricted to five or six.

*Plasma Analysis.* Plasma free fatty acids were determined by the

method of Dole and Meinertz (1960) scaled down to permit use of 0.5 ml. of plasma. The titration was performed as described by Tarrant, Thompson and Wright (1962). Glucose was determined in aliquots of 0.1 ml. using the glucose oxidase reagent of Hugget and Nixon (1957).

*Hormones.* Glucagon of porcine origin recrystallised 2x and 10x, and crystalline porcine insulin free from glucagon, were gifts from Novo Therapeutic Laboratories, Copenhagen.

*Drugs.* Reserpine and hexamethonium bromide were gifts from Mr C. Ashford, Crookes Laboratories Ltd, Park Royal, N.W.10.

*Injections.* Glucose was dissolved in 0.9 per cent w/v NaCl to yield a concentration of 30 g./100 ml. Usually 5-6 ml. of this solution was injected i.v. via the wing vein. Glucagon and insulin were dissolved in 0.9 per cent w/v NaCl solution in such concentrations that the volumes injected were below 0.5 ml.

### Results

*Glucose.* The injection of glucose (1.5 g./kg. body weight) into fed laying birds was followed by a prompt rise in the levels of plasma glucose which quickly returned to the normal values within 30 min. No change was noted in the levels of plasma FFA (Fig. 1). With starved immature birds, in which the levels of plasma FFA are increased above those in the fed immature bird (Heald and Rookledge, 1964), injection of glucose also produced little major effect on the levels of plasma FFA (Fig. 2). The clearance of glucose was obviously extremely rapid and in an attempt to maintain a higher concentration for a longer period of time glucagon was administered.

*Glucagon.* Intravenous injection of glucagon (0.2 g./kg.) was followed by a rapid increase in both plasma glucose and FFA. This occurred in birds which were in lay or immature and was independent of whether the birds had been fed or starved (Table 1). The response in the plasma glucose in starved birds was less than in fed birds presumably owing to a lowered level of liver glycogen as a result of starving (Hazelwood and Lorenz, 1959).

*Insulin.* When insulin (1.0-1.5 i.u./kg.) was administered the levels of plasma glucose decreased as expected. However, simultaneously there was a rapid and large increase in the levels of plasma FFA. This is illustrated in Fig. 3 for a group of fed immature birds. It will be noted that the levels of plasma FFA rose promptly as the levels of plasma glucose decreased, returning to the normal values when plasma glucose also returned towards normal. This effect was also found in starved birds. Increasing the dosage of insulin merely prolonged the effect (Fig. 4). Thus, with a dose level of 50 i.u. kg. plasma FFA remained elevated for up to 4.0 hr. (the duration of the experiment) while the plasma glucose remained substantially depressed. At no time were the birds unconscious or showed signs of convulsive activity.

*Reserpine, Hexamethonium and Response to Glucagon and Insulin.* The results obtained above were contrary to those obtained with mammalian species and in seeking an explanation, adrenergic stimulation was considered as a possibility. This was also prompted by the observation that

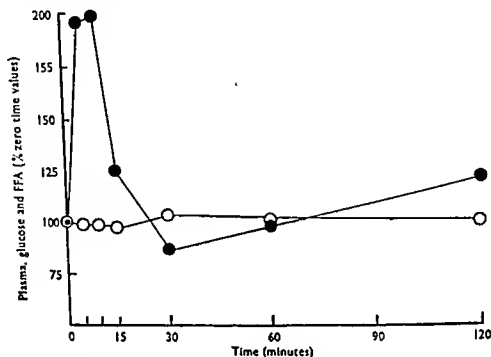


FIG. 1. Effect of injecting glucose (1.5 g./kg. body weight) on plasma FFA and glucose levels of fed laying birds (30-35 weeks of age). Each point is an average of 4 birds. ●—● plasma glucose; ○—○ plasma FFA. Initial absolute values were: glucose 220 mg./100 ml. (179-252). FFA 1,880 $\mu$  equiv./litre (1,220-3,760).

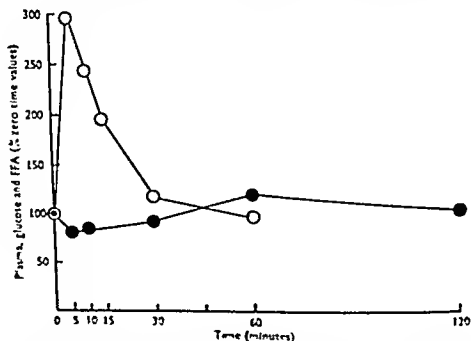


FIG. 2. Effect of injecting glucose (1.5 kg./body weight) on the plasma FFA and glucose levels in immature starved birds (aged 13-16 weeks). Each point is the mean from 3 birds. ●—● plasma glucose; ○—○ plasma FFA. Initial absolute values were: glucose 238 mg./100 ml. (226-257). FFA 872 $\mu$  equiv./litre (615-1,190).

TABLE I  
*The effects of glucagon on plasma FFA and glucose in laying and immature birds*

Glucagon (0.2 mg./kg.) was administered i.v. immediately after withdrawal of the zero time sample. Laying birds were 29-35 weeks old. Immature birds were 12-15 weeks old. Values are given as percentage zero time samples.  $\pm$ S.E.M. Groups ranged from 3-7 birds/group. Starved birds were given water only for 24 hr.

Time after injection (hr.)	Physiological state					
	In lay			Immature		
	Fed		Starved	Fed		Starved
0	Glucose	FFA	Glucose	Glucose	FFA	Glucose
0.25	—	—	100	100	100	100
0.5	146.1 $\pm$ 10.7	240.1 $\pm$ 19.1	116.5 $\pm$ 2.0	148.0 $\pm$ 8.6	347.0 $\pm$ 35.0	116.0 $\pm$ 16.5
1.0	146.0 $\pm$ 1.3	215.7 $\pm$ 15.0	128.2 $\pm$ 1.8	125.0 $\pm$ 15.9	266.0 $\pm$ 9.0	130.0 $\pm$ 8.8
2.0	141.1 $\pm$ 6.8	125.2 $\pm$ 19.6	103.6 $\pm$ 1.7	116.0 $\pm$ 6.8	110.0 $\pm$ 8.1	110.0 $\pm$ 4.9
4.0	117.0 $\pm$ 6.8	160.0 $\pm$ 14.0	102.0 $\pm$ 0.5	103.0 $\pm$ 3.8	89.6 $\pm$ 8.1	100.0 $\pm$ 4.6
			105.1 $\pm$ 2.7	—	134.0 $\pm$ 24.1	96.6 $\pm$ 8.6
						88.5 $\pm$ 10.6
						86.7 $\pm$ 7.25

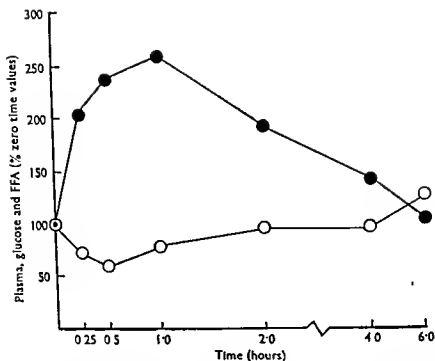


FIG. 3. Effects of insulin (1.5 i.u./kg. on plasma FFA and glucose of fed immature birds aged 13-16 weeks. ●—●, plasma FFA; ○—○ plasma glucose. Initial absolute values were: glucose 216 mg./100 ml. (211-236) FFA 339 $\mu$  equiv/litre (76-555).

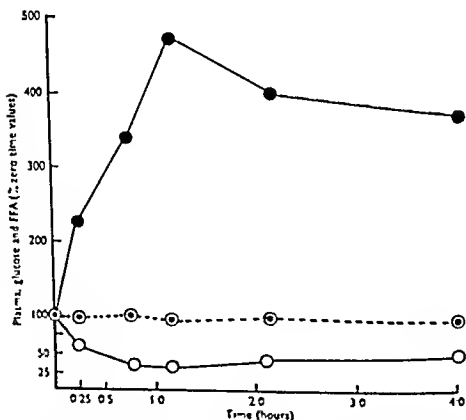


FIG. 4. Effect of insulin (50 i.u./kg.) on plasma FFA and glucose levels in fed immature birds (age 16 weeks). Each point is the average of three birds. ●—● and ○—○, plasma FFA and glucose levels in birds receiving insulin; ●— — ● and ○— — ○, plasma FFA and glucose levels in birds receiving an equivalent amount of valine. Absolute starting values: see Figs. 1-3.

injection of glucagon was followed by a gasping response lasting 2.5 min. In mammals it has been shown that glucagon or insulin induce a release of catecholamines from the adrenal medulla (Bethune, Goldfien, Zileli and Despointes, 1957; Sarcione, Back, Sokal, Mehlman and Knoblock, 1963) into the blood stream. In the fowl, the major adrenal catecholamine is *nor*-adrenaline (Shepherd and West, 1951) and since infusion of *nor*-adrenaline in the dog has been found to induce increased

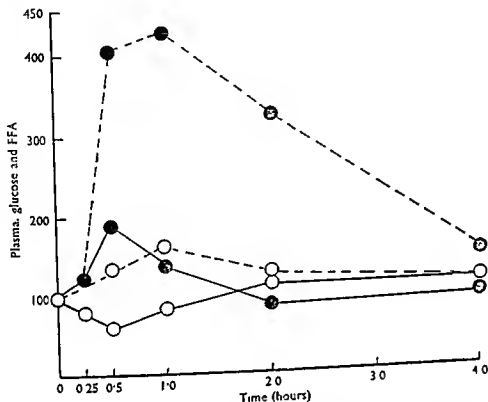


FIG. 5. Effects of insulin (1.5 i.u./kg.) and glucagon (0.2 mg./kg.) on the plasma FFA and glucose in immature fed birds (aged 16 weeks) previously treated with hexamethonium bromide (5 mg./kg.) ●—● and ○—○ plasma FFA and glucose in birds receiving insulin; ●- -● and ○- -○, plasma FFA and glucose in birds receiving glucagon. Absolute starting values were: see Figs. 1-3.

levels of plasma fatty acids (Feigelson, Pfaff, Karmen and Steinburg, 1961) it was thought possible that a similar response to *nor*-adrenaline might be occurring in the fowl.

Direct intravenous injection of *nor*-adrenaline (0.2 g. kg.) though increasing plasma glucose by 25 per cent in 30 min. (cf. Miall, 1958) and evoking a gasping response did not induce any increase in the plasma FFA. However, such a result could mean simply that the catecholamine was destroyed before reaching an effective concentration in the adipose tissue. Attempts were therefore made to deplete the adrenals of catecholamines before injecting glucagon or insulin. It has been shown (Burak, Weiner and Hagen 1960; Schuman, 1958) that the adrenal catecholamines of the adult fowl are depleted by 90-93 per cent within 5 days of an intramuscular injection of reserpine (10 mg./kg. body weight). Laying and immature birds were treated

with reserpine and after 5 days were injected with glucagon or insulin. The results (Table 2) showed that this treatment did not affect the pattern of the response which was identical with that previously found in birds not receiving reserpine.

In these experiments it was assumed that reserpine also depleted the catecholamines in the nerve endings in the adipose tissue. Though this has been shown to be true for the rat (Paoletti, Smith, Maickel and Brodie, 1961) a similar effect has not yet been demonstrated in the bird and an alternate approach, prevention of the release of *nor*-adrenaline, was therefore used employing hexamethonium bromide as a blocking agent.

Birds were treated intravenously with hexamethonium bromide (5 mg./kg.) 30 min. before a further dose (5 mg./kg.) was given i.v. together with glucagon or insulin. The levels of plasma FFA rose promptly and to as great an extent as in untreated birds and returned to normal within 2-3 hr. after the injection (Fig. 5).

### Discussion

The results described above raise several points some of which may be briefly mentioned.

First, it seems clear that the domestic fowl responds to glucagon or insulin in a manner totally different from that so far generally described for the mammalian species, though a careful examination of the literature regarding mammals reveals certain indications of a possibly similar though quantitatively smaller response. Thus, although injection of insulin in man, dog or rabbit leads to a prompt decrease in the levels of plasma glucose and FFA (Dole, 1956; Bierman, Dole and Roberts, 1957; Bierman, Schwartz and Dole, 1957; Laurell, 1957; Armstrong, Steele, Altzuler, Dunn, Bishop and De Bodo, 1961), insulin, if infused at a rate of 0.05-0.2 i.u./kh./min. into the dog leads to an initial depression in plasma FFA followed by an increase. Further, glucagon which *in vitro* stimulates the release of FFA from mammalian adipose tissue (Steinberg, Shafir and Vaughan, 1959; Hagen, 1961) also induces a rapid transient rise in plasma FFA when administered to patients with hepatitis (Drieling, Bierman, Debos, Elsbach and Schwartz, 1962).

The results with birds treated with reserpine or hexamethonium suggest strongly that catecholamine release plays little, if any, part in the increased plasma FFA. Indeed when this work had been completed, Carlson, Liljedahl, Verdy and Wirsén (1964) reported that the steady intravenous infusion of *nor*-adrenaline in the fowl failed to increase the levels of plasma FFA though the systolic pressure was greatly increased. Further, these authors showed that adrenaline or *nor*-adrenaline did not stimulate a release of FFA from avian adipose tissue *in vitro*. Similar results were found by S. Hart in these laboratories. Such results



TABLE 2

*The effect of reserpine treatment on the response of laying and immature birds to glucagon and insulin.*

Birds were given reserpine (10 mg./kg. im.) 5 days before injection of glucagon (0.2 mg./kg.) or insulin (1.5 i.u./kg.). Values are given as percentage of the zero time values  $\pm$  standard error. Eight immature birds were used with glucagon, and three with insulin. Three laying birds were used to test the effects of glucagon. Immature birds were 12-15 weeks of age, mature birds 35 weeks of age.

Substance tested	Physiological state	Quantity determined	Time after injection					
			0	0.25	0.5	1.0	2.0	4.0
Glucagon	Immature	FFA	100	526 $\pm$ 84	511 $\pm$ 174	434 $\pm$ 57	142 $\pm$ 38	78 $\pm$ 28
		Glucose	100	151 $\pm$ 8.6	164 $\pm$ 16.7	134 $\pm$ 12.6	110 $\pm$ 3.0	116 $\pm$ 2.5
Glucagon	In lay	FFA	100	141 $\pm$ 23	195 $\pm$ 22	180 $\pm$ 14	169 $\pm$ 18	113 $\pm$ 8
		Glucose	100	109 $\pm$ 2.9	132 $\pm$ 72	151 $\pm$ 3.2	125 $\pm$ 7.8	97 $\pm$ 2.6
Insulin	Immature	FFA	100	159 $\pm$ 26	156 $\pm$ 23	184 $\pm$ 23	179 $\pm$ 23	134 $\pm$ 18.7
		Glucose	100	68 $\pm$ 6.5	53 $\pm$ 7.3	46 $\pm$ 8.4	79 $\pm$ 4.9	99 $\pm$ 3

suggest that the avian adipose tissue is relatively insensitive to catecholamines in its release of FFA.

One explanation for the increase in FFA found above could lie in the increased ability of avian adipose tissue to respond to glucagon (cf. Carlson *et al.*, 1964). The increase following insulin injection can be similarly ascribed. Thus there is considerable evidence (Berthet, 1963) that the maintenance of levels of blood glucose in the mammal is the resultant of a balance between insulin and glucagon secretion. Further, insulin hyperglycaemia is accompanied by an increased level of glucagon in the blood. Since the avian pancreas contains some 6-10 times more glucagon than other species (Vuylsteke and de Duve, 1953) a similar response of the avian pancreas to insulin could result in a release of glucagon in quantities sufficient to increase the levels of plasma FFA. Viewed in this manner glucagon, at least in the domestic fowl, could be considered to play a significant role in regulating the levels of plasma FFA. In this connection it seems reasonable to suggest that the increased levels of plasma FFA found in starving mammals (Masoro, 1962; Kronfeld, 1965) and in the starving immature bird (Heald and Rookledge, 1964) may be the result of increased glucagon secretion.

Other queries arise on consideration of the above results. Thus it may be asked why the FFA are not rapidly re-esterified as in the mammal and thus lead to a fall in the level rather than an increase? It may be suggested that the FFA in the bird do not play a role in caloric stasis similar to that in mammals or alternatively that the metabolic system of the bird, particularly when in lay, is geared to the production of yolk lipids from the circulating fatty acids and not to their conversion to depot fats or their oxidation to  $\text{CO}_2$ . It may also be suggested that the well-known resistance of the fowl to prolonged insulin hypoglycemia is due to an ability to utilise long chain FFA as a cerebral energy source, though data are lacking here.

The suggestions and speculations made above are open to direct experimentation of one type or another and have been made in conforming with the requests of the organisers of this Symposium. It seems clear, however, that studies on the fowl, by showing up major metabolic differences from mammals, could be of value, not merely in understanding the physiological processes associated with the changed metabolic response but may cast new light upon problems of considerable significance in the metabolism of other species.

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# THE MECHANISM OF PROTEIN SYNTHESIS IN THE HEN OVIDUCT

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## *Synopsis*

THE MAGNUM of the oviduct of the laying hen, when homogenised in 0.44 M sucrose, can be fractionated by differential centrifuging. The residual 600 g pellet ( $P_1$ ) obtained after extensive washing contains most of the DNA of the tissue. Centrifuging at 10,000 g gives a mixed fraction (I) most of which sedimented at first with the  $P_1$ , but was subsequently washed out of it. This fraction was very rich in RNA and incorporated amino acids very readily in isolation or in minced tissue incubations. A very small 100,000 g pellet was obtained with very small ability to incorporate amino acids. The final supernatant was rich in protein. The I particles, a mixed population, were subfractionated with parallel enrichment of RNA content and amino acid incorporating activity. The requirements for incorporation of amino acids by I particles resemble those found with the microsomes of other tissues. Nucleic acids appeared to be important in this activity. Evidence was found for the presence of radioactive ovalbumin in the particles after a short incubation of the tissue with radioactive amino acid. It is suggested that these particles are derived from structures similar in function to the endoplasmic reticulum, that they are involved in the synthesis of egg-white proteins, and that their presence represents an adaptation to the function of the tissue in secreting large quantities of protein.

## *Introduction*

It is now generally accepted that in most cells, proteins are synthesised by a particular complex of enzymes and co-factors in the cell sap and the endoplasmic reticulum. Cell sap enzymes have been shown to activate amino acids using ATP\* to form amino-acyl-AMP complexes and then to transfer the activated amino acid to a small RNA molecule

\* Abbreviations: ATP, adenosine 5' triphosphate, GTP, guanosine 5' triphosphate, PEP, phospho(enol)pyruvate, RNA, ribonucleic acid, DNA, deoxyribonucleic acid, tRNA, transfer RNA; TCA, trichloroacetic acid

(transfer RNA), there being at least one specific activating enzyme and tRNA molecule for each amino acid. Polypeptides are believed to be formed on the ribonucleoprotein particles of the endoplasmic reticulum by the transfer of the amino acid from tRNA to the growing end of the polypeptide chain. An RNA molecule called the "messenger" carries in its base sequence the information coding for the amino acid order and GTP is also required for peptide bond formation (for example see Zubay, 1963).

Some doubt exists about the operation of this system in the magnum of the hen oviduct as a result of the work of Hendler (1956, 1957, 1959, 1961, 1963a). He has shown that centrifuging a homogenate of the oviduct does not give rise to the usual spectrum of cell particles. Instead, most of the particulate material is sedimented by 600 g applied for 10 min., so that a normal microsomal fraction is not obtained. In addition, he has found that when amino acid was incorporated into oviduct cell protein *in vitro*, lipid-bound amino acids, appeared to be intermediates in that they were rapidly labelled, had high specific activity and the incorporation into them rapidly reached a plateau. On dilution or removal of the radioactive amino acid, the lipid-bound activity was rapidly lost, while incorporation into protein continued to rise. Hendler has collected evidence to show that the current hypothesis for the mechanism of protein synthesis ought not to be accepted without reservations (Hendler, 1963b) and he has proposed an alternative (Hendler, 1962).

### *Results and Discussion*

A few years ago attempts were being made to prepare very radioactive egg proteins to use in parallel with free amino acids, in studies of protein synthesis in the developing chick embryo (Carey, 1964) and an endeavour was made to extend Hendler's work in order to achieve this end. It soon became apparent that although most of the particles of an oviduct homogenate were sedimented by centrifuging at low speed, subsequent washing of the pellet removed material which possessed different properties from that remaining behind. This new fraction was itself particulate and could be sedimented by applying 10,000 g for 10 min.

In the initial stages of any study such as this it is difficult to be sure of the nature of the cell fractions being separated. In this study, the fractions have therefore been given symbols which are as follows:

Homogenate centrifuged 600 g 10 min. pellet  $P_1$  supernatant  $S_1$ ;  $S_1$  centrifuged 10,000 g 10 min. pellet  $P_2$  supernatant  $S_2$ ;  $S_2$  centrifuged 105,000 g 60 min. pellet  $P_3$  supernatant  $S_3$ . Material which appeared in the supernatants on washing  $P_1$ , if it was kept separate, is referred to as  $P_1W$ . In the later stages, all the 600 g supernatants were combined, and the sediment produced by applying 10,000 g for 10 min. was then referred to as the I particle fraction.

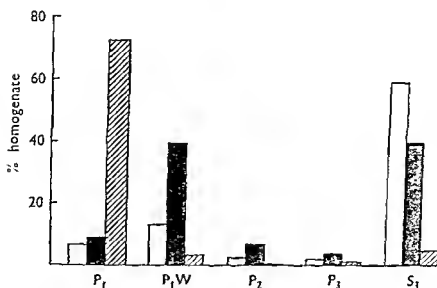


FIG. 1. The distribution of protein, RNA and DNA between cell fractions of the hen oviduct. Each entity is expressed as a percentage of the amounts in the unfractionated homogenate.

*Open bars: protein; Solid bars: RNA; Hatched bars: DNA.*

Fig. 1 shows the distribution of some properties among cell fractions obtained from the oviduct. It will be noted that most of the DNA is in the P<sub>1</sub>, and this fraction, as is usual, very likely contained the nuclei in addition to red cells and unbroken tissue. A large proportion of the RNA of the tissue which had sedimented first with the P<sub>1</sub> was washed out into the P<sub>1</sub>W, giving this fraction a high RNA:protein ratio. The RNA:protein ratios of the P<sub>2</sub> and P<sub>3</sub> were also quite high but they contained relatively small quantities of material. The S<sub>3</sub> contained a large proportion of the protein of the homogenate, a result

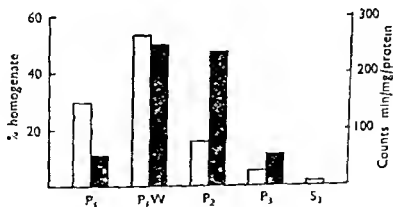


FIG. 2. The incorporation of <sup>14</sup>C-lysine by cell fractions of the hen oviduct.

*Open bars: activity in the protein of the fraction, after incubation of the minced tissue with <sup>14</sup>C-lysine for 15 min. at 37°C before fractionation, expressed as a percentage of the unfractionated homogenate.*

*Solid bars: specific activity (counts min/mg protein) of the protein after incubation of the isolated fraction with the mixture referred to in Table 1.*

which should be compared with that of the Oades and Brown (1965), indicating that 95 per cent of the magnum protein was water soluble.

It is possible that the high RNA content of the cell sap is an artefact. Large quantities of egg-white proteins appear to be present in it, and the carbohydrate groups of these are extracted along with the nucleic acids by hot TCA and subsequently interfere with the orcinol reaction for determination of RNA. Steps were taken to destroy these hexoses (Solomon, 1957) but it is possible that they were not completely successful.

Fig. 2 shows that the particles washed out of the  $P_1$  fraction were very effective in incorporating amino acids either in minced tissue incubated *in vitro* and subsequently fractionated or when incubated with radioactive amino acid and co-factors after isolation from the cell. The  $P_2$  fraction was also very active in incorporating amino acids after isolation from the cell, and in view of its low protein content it clearly has a high specific activity after incorporation by minced tissue.

It is clear that in centrifugal and biochemical properties  $P_1W$  and  $P_2$  resemble each other closely. They are found in supernatants of 600 g centrifugings, and are themselves sedimented at 10,000 g for 10 min., they have a high RNA:protein ratio and they are very active in incorporating amino acids *in vitro*, both in whole cells and after isolation from the cells. These two fractions have, therefore, been pooled (by combining all the 600 g supernatants before centrifuging at 10,000 g) and given the symbol I.

The I particles have been subfractionated in two ways. First by treatment with 0.4 per cent deoxycholate and centrifuging the clarified suspension at 105,000 g for 60 min. The pellet so obtained was about twice as rich in amino acid incorporating activity and RNA as the original I particles, and the soluble material contained more of the protein. However the RNA:protein ratio of the particles was not as high as is usually obtained with ribosomes.

Fractionation was also obtained by layering I particles in 0.44M sucrose over denser sucrose in a sector tube and centrifuging at 2,000 g for 30 min. The heavier material sedimenting through the dense sucrose contained less of the RNA and amino acid incorporating activity, whereas particles remaining in the 0.44M sucrose were richer in these properties. Preliminary evidence, obtained with the electron microscope, indicates that the heavier material contains most of the mitochondria present in the original I particles.

Although they differed from microsomes in centrifugal properties, the particles were found to resemble them in their requirements for amino acid incorporation *in vitro* (Table 1). The catalytic quantities of ATP added were not always required, but (catalytic) GTP and higher concentrations of PEP, the ATP regenerator, were required. Cell sap enzymes were also necessary. Liver cell sap was used in preference to oviduct material because of the high protein content of the latter which



might have interfered with subsequent preparative procedures. Puromycin, a recognised inhibitor of protein synthesis, was strongly inhibitory at a low concentration. Similar requirements were found with total I particles or with the ribonucleoprotein particles (Ip) derived from them by treatment with deoxycholate.

TABLE 1

*Effect of incubation conditions on the incorporation of  $^{14}\text{C}$ -lysine into protein by hen oviduct cell fractions. The isolated fractions were incubated for 30 min. at  $37^\circ\text{C}$ . in 1 ml. of a buffered salt solution containing the usual mixture of co-factors (Korner 1961) 1  $\mu\text{C}$ . of  $^{14}\text{C}$ -lysine, and 0.2 ml. of liver cell sap*

Incubation mixture	Particle preparation	
	I cpm/mg. protein	Ip cpm/mg. protein
Complete	1,080	1,160
Omit ATP	1,120	728
Omit GTP	222	194
Omit PEP	10	3
Omit, ATP, GTP, PEP	16	10
Omit liver cell sap	434	10
Omit particles	0	8
Plus $10^{-4}\text{M}$ puromycin	44	—

Amino acids linked to tRNA prepared from chicken liver were also incorporated into protein by I particles (Table 2). In this case ATP actually inhibited slightly, although no requirement need necessarily be expected. GTP was required as expected and so also was PEP.

TABLE 2

*Effects of incubation conditions on the incorporation of  $^{14}\text{C}$  amino acid linked to tRNA into protein by hen oviduct I particles. Incubation conditions were the same as those in Table 1 except that  $^{14}\text{C}$  aminoacyl-tRNA (18,300 cpm 0.15 mg.) replaced the  $^{14}\text{C}$ -lysine*

Incubation mixture	cpm/mg. protein
Complete	1,860
Omit ATP	2,200
Omit GTP	834
Omit PEP	284
Omit ATP, GTP	937
Omit ATP, PEP	338
GTP, PEP	234

This latter requirement could not be replaced by a higher GTP concentration (ten-fold increase), but this concentration of GTP was found to be inhibitory even in the presence of PEP.

Hoagland, Stephenson, Scott, Hecht and Zamecnik (1958) found with rat liver microsomes that an ATP generator was required for the transfer of amino acids from tRNA, and that this could not be replaced by increasing the GTP concentration five-fold, which is in agreement with the above observations. On the other hand, Korner (1961) has

found that rat liver ribosomes did not require an ATP generator for the incorporation of free amino acids, whereas in this study PEP was required in all the systems tested, even where, in the incorporation from free amino acids, a requirement for ATP could not be demonstrated. In the latter case it is possible that there was sufficient ATP in the enzyme preparations to supply the catalytic quantities needed for the reaction.

The involvement of nucleic acids in the incorporation is further borne out by the effect of ribonuclease, which inhibited strongly at low concentrations (Fig. 3). Lecithinase-C was much less inhibitory.

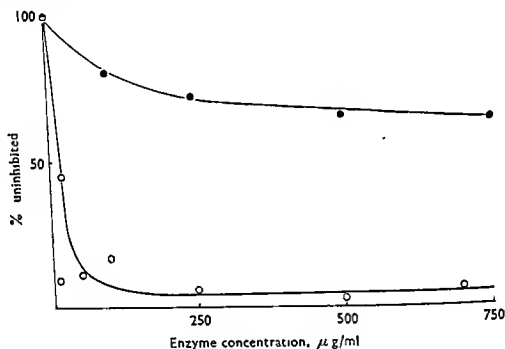


FIG. 3. The effect of lecithinase-c and ribonuclease on the incorporation of  $^{14}\text{C}$ -lysine by isolated hen oviduct I particles.

Open circles: ribonuclease; closed circles: lecithinase-c.

In addition, after the incorporation of amino acids *in vitro*, the lipid extracts of the particles were not radioactive, but the hot TCA extract (which contains the nucleic acids) was very radioactive. A radioactive lipid extract was prepared by incubating minced oviduct with  $^{14}\text{C}$ -lysine and extracting a TCA precipitate of the tissue with organic solvents. The radioactivity of this extract was not transferred to protein under the conditions used, which resemble those in which amino acid was transferred from tRNA.

These results indicate the presence in the oviduct cells of material similar to that which gives rise to the microsomal fraction in other cells. In isolation this material incorporates amino acids, and nucleic acids appear to be important in this activity. It seems likely that the mechanisms involved are similar to those found in other tissues. No evidence of the involvement of lipid-bound amino acids has been found in the cell-free preparations. This does not mean, however, that such a route does not exist in the whole cell. For example, it is possible that two routes of incorporation exist, as proposed by Hendler (1962), but that



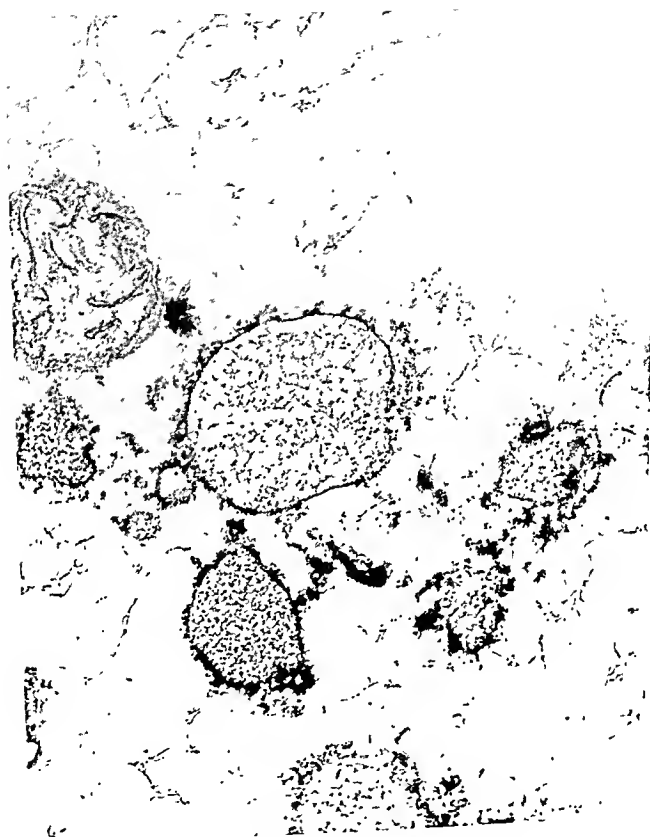
Electron micrograph of I particles fixed after isolation showing a mitochondrion, and vesicles with a limiting membrane having thin and thick regions. Most of the vesicles have structureless contents, but one has small membranous inclusions.

• 39,000



Electron micrograph of I particles fixed after isolation showing the variety of materials in the I fraction, including what are probably degenerating mitochondria in the same field as others of more normal appearance.

$\times 25,500$



Electron micrograph of I particles fixed after isolation - 30,000x

the route involving lipids does not survive homogenisation. This, of course, could also be true of tissues other than the oviduct.

An attempt has been made to see whether these particles are involved in the synthesis of egg-white protein. Minced oviduct was incubated with  $^{14}\text{C}$ -lysine for 15 min., at which time most of the activity incorporated into protein was found in the I particles. The tissue was then chilled and the I particles isolated. They were then incubated in a buffered salt solution at  $37^\circ\text{C}$ . which solubilised some of the protein and this was mixed with twice its weight of non-radioactive ovalbumin and separated on a carboxymethyl-cellulose column (Rhodes, Azari and Feeney, 1958). The peaks corresponding to ovalbumin  $A_2$  and  $A_3$  were radioactive, indicating that some of the incorporation was into material behaving as ovalbumin.

The appearance of thin sections of fixed I particles in the electron microscope is shown in Plates 13, 14 and 15. Compact rod-shaped mitochondria and some which were apparently degenerated forms were found, together with vesicles of a variety of sizes. These vesicles had structureless contents and were bounded by a membrane having thin and thick areas. They resemble structures seen by Hendler, Dalton and Glenner (1957) in whole cells of the oviduct.

Large vesicular bodies have been implicated in protein synthesis in a variety of different cells, i.e. in wheat germ endosperm (Morton and Raison, 1963), virus infected cells (Horne and Nagington, 1959; Penman, Becker and Darnell, 1964), in the abnormal plasma cells found in multiple myeloma (Rifkind, Osserman, Hsu and Morgan, 1962), in tumours (Smetana and Bush, 1963), in yeast cells which are rapidly synthesising mitochondria (Linnanc, 1964) derived from the Golgi apparatus of pancreatic cells in zymogen granule production (Caro and Palade, 1964) and in developing frogs' eggs (Ward, 1962).

The vesicles in the above tissues may not all be of the same type, and the reasons for their formation may differ, but they indicate that under a variety of special circumstances the protein synthetic apparatus of cells can be organised into discrete large bodies differing in appearance from the normal endoplasmic reticulum, but perhaps resembling it in basic structure and function. It seems likely that the structures observed in the oviduct and derived from it by homogenising are adaptations to the function of this tissue in synthesising large quantities of protein.

#### *Acknowledgements*

It is a pleasure to thank Miss Janet Sterlini and Miss Ruth Coxon for expert technical assistance. The British Egg Marketing Board have generously given a grant to defray expenses and the Board of Governors of St Thomas's Hospital provided for the purchase of an ultracentrifuge. The author is deeply indebted to Professor F. T. G. Prunty for his constant encouragement and advice.

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# CHANGES IN THE WATER-SOLUBLE PROTEINS OF THE AVIAN OVIDUCT IN RELATION TO REPRODUCTION AND FOLIC ACID DEFICIENCY

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## *Synopsis*

THE LITERATURE relating to the effects of sexual activity in the female fowl on oviduct composition is reviewed and reference is made to the effects of gonadal hormones on oviduct composition in the immature female chick.

The elaboration of egg-white proteins in the oviduct of the immature female chick treated with gonadal hormones is shown to be impaired in folic acid deficiency. Preliminary observations using paper electrophoresis are recorded which indicate that the A<sub>1</sub> ovalbumin is lower in the water-soluble oviduct proteins of chicks receiving oestrogen only as compared with oestrogen and progesterone.

Starch gel electrophoretic separations of water-soluble magnum proteins (WSMP) from the oviducts of female chicks receiving different hormone treatments and from a laying and broody hen are described.

The WSMP of female chicks receiving oestradiol alone are shown to be similar to those in the broody hen and to be lower than those in chicks receiving oestradiol plus progesterone or oestradiol plus testosterone. Electrophoretic studies indicate that a combination of gonadal hormones is required for the elaboration of egg-white proteins in the oviduct.

## *Introduction*

Since the classical work of Richardson (1935) describing histological observations and probably the first chemical examination of regions of the oviduct of the laying hen there has been limited biochemical information available in the changes in the composition of the oviduct associated with changes in the functional activity of this organ. Prior to the onset of laying in the pullet the oviduct increases in weight from about 0.1 g. to 40-60 g. This weight increase accompanies parallel changes in the growth of the ovary, and similar changes



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Starch gel electrophoretic separations of water-soluble magnum proteins (WSMP) from the oviducts of female chicks receiving different hormone treatments and from a laying and broody hen are described.

The WSMP of female chicks receiving oestradiol alone are shown to be similar to those in the broody hen and to be lower than those in chicks receiving oestradiol plus progesterone or oestradiol plus testosterone. Electrophoretic studies indicate that a combination of gonadal hormones is required for the elaboration of egg-white proteins in the oviduct.

## *Introduction*

Since the classical work of Richardson (1935) describing histological observations and probably the first chemical examination of regions of the oviduct of the laying hen there has been limited biochemical information available in the changes in the composition of the oviduct associated with changes in the functional activity of this organ. Prior to the onset of laying in the pullet the oviduct increases in weight from about 0.1 g. to 40-60 g. This weight increase accompanies parallel changes in the growth of the ovary, and similar changes

in oviduct growth may be induced in the immature pullet by the administration of gonadal hormones (Common, Rutledge and Bolton, 1947). Common *et al.* (1947) also noted that there was a synergistic effect of a combined treatment with oestradiol and testosterone on oviduct growth response. Hertz (1950) showed that there was a relationship between oviduct growth and the ratio of oestrogen to progesterone administered to immature chicks and that the formation of avidin in the oviduct depended on the administration of both hormones. Investigation of the riboflavin content of the magnum region of the oviducts of immature chicks treated with oestrogen alone or a combination of oestrogen and progesterone revealed that increased riboflavin levels resulted from the double hormone treatment (Bolton, 1952).

The above observations of increased weight response and associated changes in the avidin and flavin components would suggest that these findings may be related to the elaboration of proteins similar to those of egg white. The author became interested in this aspect of the study of the oviduct as a result of some work being undertaken to study the amino acid and nucleic acid content of sections of the oviduct of the laying hen and hormone-stimulated oviduct of the immature female chick (Brown and Jackson, 1960). In the course of this work it was found that increases in oviduct weight in the immature chick resulting from the double hormone treatment with oestrogen and testosterone as compared with oestradiol alone was accompanied by a decrease in the moisture content of the tissue and an increase in protein content. In relation to this finding, the work of Brandt and Nalbandov (1956) is of particular interest since these authors showed that full functional activity of the immature oviduct and the formation of secretory granules depended on the simultaneous administration of oestrogen and progesterone. Electrophoretic studies of water-soluble oviduct proteins by Oades and Brown (1965) substantiate this evidence and are discussed later in the present paper.

It was also found that folic acid deficiency in the immature chick led to a reduction in the protein content of hormone-induced oviduct tissues (Brown and Jackson, 1957). Since the early work of Hertz and Sebrell (1944) it had been known that folic acid deficiency resulted in a much reduced oviduct weight response to oestrogens in the immature female chick but no studies of the changes in protein content or protein composition had been carried out.

Recently, however, there has been a considerable amount of research conducted using minces of tissue from the magnum region of the oviduct of the laying hen with a view to studying the elaboration of egg-white proteins. Mandeles and Ducay (1962) showed that ovalbumin and conalbumin are formed at the same rate and that the site of formation is in the oviduct tissue—an observation suggested by the earlier work of Smith, Winget and Hage (1957). Recent work on

particulate fractions of chicken oviduct tissue by Carey and Sterlini (1964) is in accord with this viewpoint. Chromatographic techniques were used by Mandeles and Ducay (1962) who showed that water-soluble oviduct proteins contained lysozyme as well as ovalbumins and conalbumin. Electrophoretic techniques were used by Williams (1962) to examine the conalbumin of egg-white protein and showed that the conalbumin fraction contained three proteins.

The work described by the author in the present contribution relates specifically to two aspects of the factors affecting water-soluble oviduct proteins. Firstly, the effect of folic acid deficiency on the levels of water-soluble proteins in whole oviduct tissues of immature chicks is considered and part of this work has been described by Brown and Badman (1965). Secondly, results are presented of a more general study of water-soluble oviduct and magnum proteins in the laying hen, the broody hen and the immature chick treated with various combinations of gonadal hormones.

## Effects of Folic Acid Deficiency on the Water-soluble Oviduct Proteins (WSOP)

### *Experimental Methods*

In the initial experiments the effects of oestrogen and oestrogen and testosterone or progesterone were examined to ascertain the levels of WSOP in the oviduct tissues. Light Sussex  $\times$  Brown Leghorn female chicks were used for these experiments while in the later experiments in which the effect of folic acid deficiency was examined, female Light Sussex chicks were used. In the hormone experiments the chicks were reared on a commercial diet to 30 days of age, and, after randomisation on the basis of initial liveweight, were allocated to three treatments which included five intramuscular injections of hormones on alternate days as follows:

- Treatment 1: 5.0 mg. oestradiol dipropionate (total dose)
- Treatment 2: 5.0 mg. oestradiol dipropionate + 5.0 mg. progesterone (total dose)
- Treatment 3: 10.0 mg. oestradiol + 5 mg. testosterone propionate (total dose)

In the case of the folic acid deficiency experiments chicks were reared on a commercial diet for 17 days and then placed on a folic acid-deficient diet for a further 14 days as described by Brown (1954). At the end of this period three treatments of five intramuscular injections on alternate days were given as follows:

- Folic acid-deficient 5  $\times$  2.0 mg. oestradiol dipropionate + 5  $\times$  1.0 mg. testosterone propionate (total dose)
- Control 5  $\times$  0.5 mg. pteroylglutamic acid + 5  $\times$  2.0 mg. oestradiol dipropionate + 5  $\times$  1.0 mg. testosterone (total dose)

Following these treatments, the oviducts were dissected out and dried *in vacuo* over  $P_2O_5$  and then ground to a powder. Fifty mg. samples of dried oviduct tissue was shaken for 1 hr. with 2.0 ml. water to extract the soluble proteins. These extracts were centrifuged at 2,500 r.p.m. for 20 min. to produce a clear supernatant which was decanted and stored at 0°C. prior to electrophoresis and nitrogen determination. Standard solutions of ovalbumin, conalbumin and lysozyme (Nutritional Biochemicals Ltd) were prepared at a concentration of 10 mg./ml. For comparison a water solution of egg white was prepared by diluting the white with an equal volume of water. Paper electrophoresis was carried out by the method of Durum (1951). The pattern of egg-white proteins was similar to that obtained by Evans and Bandemer (1956). The patterns obtained with pure conalbumin, ovalbumin and lysozyme were used as reference proteins in the identification of the egg white and water-soluble oviduct proteins. Since the conditions of electrophoresis were similar to those of Evans and Bandemer (1956) the ovoglobulin plus ovomucoid peak was identified by analogy with the electrophoretic patterns obtained by these workers. These two proteins are not resolved by this method.

### Results

It was found that ovalbumin and conalbumin peaks together with the combined peak for ovoglobulin and ovomucoid were present in the extracts from the oviducts of birds treated with oestradiol alone as well as in those from birds treated with both hormones. The levels of

TABLE 1

*Relative amounts of various water-soluble proteins separated by electrophoresis from whole oviduct tissue of chicks treated with gonadal hormones*

Hormone treatment	Control				
	Albumins			Ovomucoid + ovoglobulin	Conalbumin
	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>		
5.0 mg. oestradiol	93	40	97	74	150
10.0 mg. oestradiol	85	153	130	52	160
+ 5.0 mg. testosterone					
5.0 mg. oestradiol	144	78	154	71	197
+ 5.0 mg. progesterone					

hormone administration used in this experiment were comparable to those used in the histological studies of Brant and Nalbandov (1956). There were quantitative differences in the total water-extractable proteins between the different treatments, a larger amount of protein being extracted from the oviduct tissues of the chicks treated with both hormones. The data in Table 1 illustrate this point in that the peak areas attributable to the ovalbumins and conalbumins are lower in the case of oestradiol treatment only.

The most interesting effect of folic acid deficiency was the very great reduction in the percentage of water-soluble proteins in the hormone-stimulated oviducts from deficient chicks. This accounted for the large quantitative difference in the peak areas of the WSOP from the normal and deficient chicks as shown in Table 2. Examination of the electrophoretic patterns showed that there was a very large reduction in the albumin fractions.

TABLE 2

*Relative amounts of various water-soluble proteins separated by electrophoresis from whole oviduct tissue of normal and folic acid-deficient chicks treated with gonadal hormones*

Ovalbumins	Control	Folic acid-deficient
$A_1$	81	} 34
$A_2$	66	
$A_3$	27	
Ovoglobulin + ovomucoid	29	11
Conalbumin	93	35

### Discussion

The results of the experiments on the hormonal effects suggest that there is a similarity in the electrophoretic pattern of WSOP from oviducts of hormone-treated immature chicks and that of egg white proteins. However, there appears to be a quantitative difference in the levels of water-soluble proteins formed in that the oviducts stimulated by oestrogen alone contain smaller quantities than those stimulated by a combination of oestradiol and progesterone or oestradiol and testosterone. This suggests that the histological evidence of Brandt and Nalbandov (1956) relating to the magnum region of the oviduct would be well worth verifying using electrophoretic techniques; and more precise evidence on this aspect is presented in the next section.

Though the resolution of the  $A_1$ ,  $A_2$  and  $A_3$  albumins was not very satisfactory by the paper technique, there was an indication that the  $A_1$  albumin was present in greater amount in the oviducts from the birds receiving oestrogen and progesterone and this is confirmed in later work using starch-gel electrophoresis. Lysozyme was not detected in any of the extracts but this requires further confirmation.

In view of the specific effect of folic acid deficiency on the growth of the oviduct it is interesting to note that this effect is also accompanied by a reduction in the elaboration of certain water-soluble egg-white proteins. The effects noted were not due to inanition *per se* since the folic acid-treated chicks and deficient chicks were pair fed.

## Reproductive State and Hormonal Influences on the Water-soluble Oviduct Proteins

### *Introduction*

In the previous section it was noted that the double hormone treatment of normal immature chicks resulted in increased WSOP but it was not possible to establish very precise levels of the individual albumins by the paper electrophoretic method. For this reason a more exhaustive study of the WSOP and water-soluble magnum proteins (WSMP) of the oviduct of a laying hen was carried out using the discontinuous buffer system of Poulik (1957) as modified by Lush (1961) and the gradient elution from C M-cellulose columns described by Rhodes, Bennett and Feeney (1959). Baker and Manwell (1962) made a comprehensive study of egg-white proteins and obtained positive resolution on starch-gel of lysozyme, esterase, conalbumin, mucoid, albumin and flavoprotein and tentatively of the mucin-globulin fraction. In the present comparative study of the WSOP of hens, hormone-treated chicks and egg-white proteins, identification of the proteins was by comparison with the patterns observed by Baker and Manwell (1962).

### *Experimental Methods*

To compare the WSMP and those of other regions of the oviduct of the laying hen with egg-white proteins a laying pullet was killed immediately after laying in order to avoid the presence of a yolk in the oviduct (Warren and Scott, 1935). The whole oviduct was dissected out and weighed and each region separated. The magnum region was sampled longitudinally into four equal 10 cm. lengths and dried over  $P_2O_5$  *in vacuo*. The water extracts were dialysed at 4°C. for at least 2 days against pH 4.0 ammonia-acetic acid buffer prepared from 0.1M ammonia and 0.1M acetic acid. The resulting precipitate of crude ovomucin was centrifuged down and a clear dialysate was used for protein separations by starch-gel electrophoresis and carboxymethyl cellulose (CM-cellulose) column chromatography.

While it appeared that CM-cellulose chromatography was useful for qualitative comparison of samples it was not satisfactory for quantitative work since the recovery of proteins based on protein determinations by the method of Lowry, Rosenbrough, Farr and Randall (1951) showed protein recovery to be incomplete. The starch-gel electrophoresis was carried out on a quantitative basis by strictly standardising the conditions of electrophoresis and protein staining.

It was decided to use larger chicks than had been used in the work described in the previous section and to compare the WSMP from chickens treated with oestrogen alone and in combination with either progesterone or testosterone with the WSMP from a laying hen and a broody hen. The dose levels of gonadal hormones were similar to those

used in the previous work. Chicks of approximately 700 g. body weight were used. Comparisons of WSMP were made by carrying out starch-gel electrophoresis under standard conditions and scanning the runs in an automatic densitometer. Relative amounts of A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub> albumins and of an intermediate post-albumin band were established by rescanning on an extended scale.

### Results

The results of the examination of the water-soluble proteins of the various sections of the oviduct of a laying hen are given in Table 3.

TABLE 3

*Water-soluble protein content of regions of the oviduct in a laying hen*

Oviduct section	Weight (g.)	Per cent protein in dry matter	Water soluble per cent of total protein	Crude ovomucin per cent of total protein	Total water-soluble protein (g.)
Total oviduct	57.3	—	—	—	8.28
Funnel	1.8	38.9	42.4	Nil	0.07
Magnum	33.4	60.3	94.9	2.8	6.35
Isthmus	6.0	56.9	45.0	Nil	0.36
Uterus	11.6	51.1	29.7	3.2	1.45
Vagina	4.5	51.5	21.2	3.0	0.05

The magnum contributed the largest amount to the total water-soluble protein but there was a considerable contribution by the soluble proteins of the uterus. There was a very high percentage (approx. 95 per cent) of water-soluble protein in the magnum region. The funnel and the isthmus regions also contained a large quantity of water-soluble protein. A crude ovomucin fraction was not separated from the funnel or the isthmus regions.

The starch-gel electrophoretic pattern of the water-soluble proteins from the five sections of the oviduct of the laying hen showed the uterus contained a fairly large quantity of water-soluble proteins, but the proteins were not egg-white proteins except for possible traces of ovalbumins. The major component of the uterus was found to migrate to a position immediately behind ovalbumin A<sub>3</sub>, the slowest moving of the three albumins. This protein was also found to be a major component of the water-soluble extract of the vagina and funnel. The electrophoretic patterns of the magnum were very similar to those of egg-white proteins both quantitatively and in the relative amounts of each protein present, although there was an increased concentration of the immediate post-albumin band. Lysozyme was not detected satisfactorily in magnum extracts by starch-gel electrophoresis.

The precipitates from the dialysate of egg-white proteins when dissolved in borate buffer and subjected to electrophoretic separation were found to contain many egg-white proteins including ovalbumins,



conalbumins, lysozyme and globulins, in agreement with the observations of Rhodes, Azari, and Feeney (1958), and those of Feeney, Ducay, Silva and MacDonnell (1952). It was found that considerable trailing occurred in the electrophoretic pattern from the precipitate, suggesting a range of molecular weights or denaturation of proteins, and a large proportion of the protein was immobile.

If unblended egg-white complete with chalazae was dialysed against the pH 4.0 ammonia-acetic acid buffer, the crude ovomucin precipitate developed initially round the chalazae and eventually appeared to be concentrated in close proximity to the chalazae. This effect would

TABLE 4

*Oviduct weight, dry matter and water-soluble protein content of magnum in a laying hen, broody hen and female chicks treated with gonadal hormones*

Source of oviduct tissue	Oviduct weight (g.)	Magnum weight (g.)	Per cent dry matter of magnum	Water-soluble protein as per cent of total protein
Laying hen	61.4	29.3	31.9	98.2
Broody hen	15.0	5.8	16.7	49.0
Immature female chicks (700 g. body weight) receiving:				
50 mg. oestradiol (A)	13.3	2.6	21.9	50.5
100 mg. oestradiol	15.0	5.8	23.6	79.4
+ 50 mg. testosterone (B)				
50 mg. oestradiol	6.3	2.5	29.7	82.0
+ 50 mg. testosterone (C)				

oviduct growth and the synergetic effect of oestrogen and androgen. Though the total oviduct weight in the chicks treated with oestrogen and progesterone were much lower than those of chicks receiving oestrogen alone, the weight of the magnum region was only slightly lower. It is also interesting to note that oestrogen in combination with progesterone also resulted in an increase in the magnum dry matter percentage as a result of increased synthesis of protein in this region of the oviduct. Similarly, the synergetic effect of testosterone, in addition to producing the largest oviducts, also led to an increase of magnum size to a level almost twice that in the other two treatments. The dry matter contents and WSMP levels on this treatment were also as high as those in the chicks receiving oestrogen and progesterone.

The percentage of WSMP in the total magnum protein in the laying hen was slightly greater than that in any of the hormone-treated chicks and approximately twice that in the magnum of the broody hen. The percentage WSMP of the total magnum protein of the broody hen was comparable to that in birds receiving oestrogen only. A high percentage (58.6 per cent) of the WSMP of the broody hen was precipitated on dialysis as crude ovomucin as compared with only 2.9 per cent in the laying hen and 4.1 to 7.1 per cent in the birds receiving the two double hormone treatments. In the case of the chick receiving oestrogen only, 35.2 per cent of the WSMP was precipitated on dialysis.

The total amount of WSMP of the laying hen just after laying was sufficient to supply the egg-white proteins present in two eggs and would explain the approximate delay of 2 days in the appearance of labelled protein in egg white after the intravenous injection of radioactive amino acids (Siva Sanker and Theis, 1959; Mandeles and Ducay, 1962). The dry-matter percentage of about 30 of the WSMP in the laying hen was about three times as high as in egg white, supporting the hypothesis that water is added to the egg-white proteins lower down the oviduct (Smith, Hoover, Nordstrom and Winget, 1957).

The relative amounts of the various proteins in the WSMP separated on starch gel are given in Table 5. The pattern of egg white and WSMP of the laying hen were similar and the WSMP of the broody hen were dominated by material that was not separated into zones and appeared as a trail from the origin to the position of the conalbumins. Apart from this, the major component is the immediate post-albumin band and ovalbumins were present in small quantity and ill-defined. All three ovalbumins were present in the WSMP of all the hormone-treated chicks. Scans from separations of WSMP in chicks given oestrogen and androgen showed good definition of peaks and the main differences compared with the laying hen related to an increased relative amount of A<sub>2</sub> and A<sub>3</sub> ovalbumins together with an increased level of the post-albumin fraction (see Table 5). The magnitude of the post-albumin band appeared to be at a maximum in the broody hen and the chick treated with oestrogen only and to decrease in the chicks treated with

oestrogen and testosterone. There was also a decrease in chicks treated with oestradiol and progesterone to a minimum in the laying hen and egg-white proteins. Conalbumin was present in all WSMP examined including those from the broody hen.

TABLE 5

*Relative amounts of water-soluble proteins separated by starch-gel electrophoresis from egg white and magnum sections of a laying hen, broody hen and chicks receiving gonadal hormones*

(Experiment 2)

Protein fraction	Ovalbumins				P <sub>5</sub>	P <sub>6</sub>	P <sub>7</sub>	Con- albumins	P <sub>8</sub>	Lysozyme
	A <sub>1</sub>	A <sub>2</sub>	A <sub>3</sub>	P <sub>4</sub>						
Dialysed egg white	24.5	17.0	5.6	0.8	10.6	3.8	4.3	2.91	3.5	0.9
Laying hen	21.0	24.2	5.6	1.6	7.3			30.6		0
Broody hen	1.4	6.6	3.9	19.5	5.0			63.6		0
5.0 mg. oestradiol (A)	33.6		3.4	12.3	6.7			37.2	6.8	0
10.0 mg. oestradiol + 5.0 mg. testosterone (B)	9.1	16.5	12.3	3.8	12.4			38.0	8.6	0
5.0 mg. oestradiol + 5.0 mg. progesterone (C)	13.4	30.0	10.1	5.8	6.0			24.1	9.2	0

Peaks P<sub>5</sub>, P<sub>6</sub> and P<sub>7</sub> are not separated except in dialysed egg white. Peak P<sub>8</sub> has been described as the post-conalbumin fraction (Baker and Manwell, 1962).

### Discussion

The results of the present investigation are described in more detail by Oades and Brown (1965). Starch-gel electrophoresis of WSOP of the fowl appears to be a useful tool for the study of the effect of natural reproductive state and hormonal modification of oviduct development on the relative percentage of egg-white proteins in the oviduct. The data for the composition of the magnum section of the oviduct show that changes in this region are influenced preferentially by natural ovarian activity or by certain combinations of gonadal hormones in the immature female.

The present results show that a large percentage of WSOP is located in the magnum and further that alteration of the WSMP of the oviduct may be induced by hormone administration. The enhanced elaboration of egg-white proteins in the combined oestrogen and progesterone treatment corresponds with the observation of Hertz (1950) concerning the elaboration of avidin and the report of Bolton (1952) on the formation of riboflavin in the oviduct under the influence of this double hormone treatment. It is well known that a combination of oestrogen and testosterone induces changes in the metabolism of the immature fowl similar to those encountered prior to the onset of lay

in the pullet (Common, Rutledge and Bolton, 1947). It may not be surprising, therefore, that this treatment also produced increases in the WSMP similar to those in the magnum of the laying hen. The histological data of Brandt and Nalbandov (1956) indicated that a combination of oestrogen and progesterone or of oestrogen and testosterone was required to produce albumin-secreting granules in the magnum of the immature pullet. In the present work it was found that in the broody hen and in the chicks treated with oestradiol only there was a large amount of post-albumin protein in the WSMP. Furthermore, the amount of WSMP in the broody hen and the chicks treated with oestradiol only was much lower than in the laying hen or the chicks receiving treatment with both hormones. These results would support the findings of Brandt and Nalbandov (1956). There are, however, differences between the WSMP of the broody hen, laying hen and egg-white proteins and the WSMP of the chicks treated with both hormones which remain to be explained. For example, the relative increases in the concentration of albumins  $A_2$  and  $A_3$  and of the post-albumin band in the hormone-treated chicks and in the broody hen compared with that in the WSMP of laying hen or egg white suggests that the process of phosphate removal described by Perlmann (1955) from  $A_2$  may determine the origin of  $A_1$  from  $A_2$ ,  $A_2$  from  $A_3$  and of  $A_3$  from the immediate post-albumin protein. Support for this is given by the finding that there is a much lower content (50 per cent) of WSMP in the oviducts of the broody hen and chick treated with oestrogen only than that in the laying hen or chick given the double hormone treatment (80-90 per cent). Concomitant progesterone administration to the immature chick decreases the relative amount of the post-albumin protein and this effect would not support the view that broodiness in the fowl is associated with an increase in endogenous progesterone production.

There appears to be no doubt that the release of egg-white albumins requires the simultaneous effect of oestradiol combined with either testosterone or progesterone in the immature chick. Siva Sanker and Theis (1959) and Smith, Court and Martin (1959) have described a protein precursor of egg-white albumins which may well be the protein of the post-albumin band encountered in the present electrophoretic studies. These latter authors have also suggested that the release of the ovalbumins is controlled by the state of the ovary in the laying hen and that the amount of the protein precursor will decrease and the ovalbumins increase as ovulation becomes imminent.

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# PROTEIN DIGESTION AND METABOLISM IN THE COLOSTOMISED LAYING HEN

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## *Synopsis*

RESULTS are presented to show that colostomised hens are not physiologically abnormal with regard to nitrogen excretion. Using colostomised hens the metabolic faecal nitrogen (MFN) output was determined directly by feeding a nitrogen-free diet and indirectly by extrapolating to zero a regression of faecal N output against per cent nitrogen in the diet. The values obtained were 158 mg./100 g. dry-matter intake and 116 mg./100 g. dry matter consumed respectively.

The MFN values were used to calculate true digestibilities for a number of protein sources fed at different levels of dietary intake. The source of protein and the level of protein fed did not affect true digestibility. The overall mean value obtained was 88.5 per cent.

Using biological values previously determined and the digestibilities determined in this paper a factorial method was used to calculate the daily protein requirement of a laying hen. For a hen of 2 kg. body weight, laying 60 g. eggs at 75 per cent production, a daily requirement of 13.3 g./day was found.

## *Introduction*

Almost all the estimates of true and apparent digestibility of protein in poultry have been made by indirect techniques. This is because it is not easy to separate the urine and faeces which are excreted from the cloaca in a mixed form. For the determination of digestibility two methods exist. The first is by chemical separation of the faecal nitrogen from the mixed excreta, the second by surgical modification of the bird to provide a separate artificial anus.

In early work Fields and Ford (1900) determined the faecal protein directly as albuminoids and assumed this to be the total faecal nitrogen. This approach was also used by Ekman, Emanuelson and Fransonn (1919) in their protein precipitation procedure. A second approach to the chemical estimation was to determine the urinary nitrogen and obtain the faecal nitrogen by difference. Coulson and Hughes (1930)

obtained pure urine by a urethral cannula and found uric acid and

*Physiology of the Domestic Fowl* edited by C. Horton Smith and E. C. Amoroso (B.E.M.B. Symposium No. 1; Oliver and Boyd, Edinburgh and London, 1966).

# CORRIGENDA

to Paper 16, 'Protein Digestion and Metabolism in the Colostomised Laying Hen' by E. Squance, pp. 146-154.

P. 146, line 13: *for 158 read 164*

line 14: *for 116 read 207*

line 23: *for 13.3 read 12.25*

P. 147, line 8: *for differences read difference*

line 9: *for Jeffrey read Jeffay*

P. 148, Table 2: *replace entire Table by new Table 2 given overleaf*

line 14: *for consumed/day on read consumed on*

line 16: *for at zero intake read at zero nitrogen intake*

line 17: *for 116 mg. N read 207 mg. N*

lines 17 & 18: *for regression and mean daily feed intake was significantly lower than read regression was not significantly different from*

line 19: *for 158 mg. read 164 mg.*

lines 19-22: *delete entire sentence from 'This difference to feeding conditions*

line 31: *for confined read confirmed*

P. 149, Fig. 1: *replace by new Fig. 1 on p. iv.*

P. 150, line 5: *for ratio in egg production read ratio on egg production*

Table 3: *replace entire Table by new Table 3 given on p. iii.*

line 20: *for 88.5 read 90.6*

line 32: *for 95 read 93*



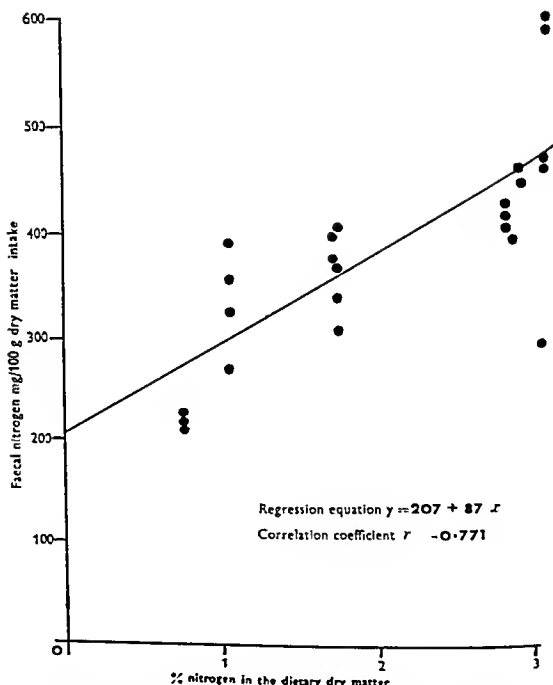
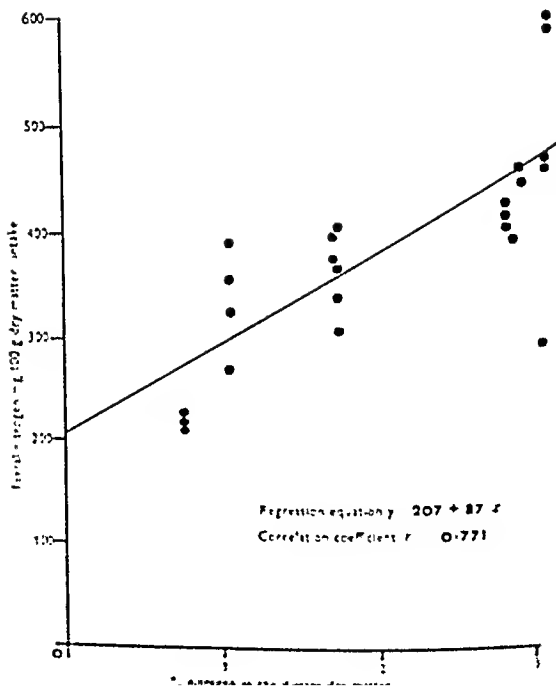


FIG. 1. Determination of MFN by extrapolation. Regression of Faecal N/100 g. D.M. ( $y$ ) on percentage nitrogen in the diet dry matter ( $x$ ). (Each point one 4-day trial period in one hen.)



obtained pure urine by a urethral cannula and found uric acid and ammonia to be 80 per cent of the total urinary nitrogen. This factor was used by St John, Johnson, Carver and Moorc (1932) and by MacDonald and Bose (1944) who took uric acid nitrogen plus ammonia nitrogen  $\times 1.25$  as the urinary component. Katayama (1924) successfully obtained one operated hen and from it calculated a widely used formula relating uric acid-N to total urinary-N and faecal-N was obtained by differences.

This work was re-examined by O'Dell, Woods, Laerdal, Jeffery and Savage (1960), who found urinary  $N = 1.096$  (uric acid N + ammonia-N). Both formulae were then applied to mixed excreta from normal hens.

TABLE 1

*Comparison of normal and colostomised laying hens on same nitrogen intake. (N in constituents as percentage of total N; mean of three determinations)*

	Total N (mg./g. D M)	Uric-N	Ammonia-N	Urea-N	Creatinine-N
Normal	77.27	51.03	2.634	0.963	1.46
Colostomised	78.63	54.96	1.724	0.741	2.60

For the direct determination the normal method is to exteriorise the rectum. Recently Ariyoshi and Morimoto (1956) and Fussell (1960) have described successful operations. O'Dell *et al.* (1960) also used this method, while Laerdal, Newberne, Savage and O'Dell (1957) used exteriorised ureters in 4-week-old male chicks. Ariyoshi (1957), Morimoto, Kubota and Ariyoshi (1961a) and the author have obtained direct determinations of digestibility on modified hens.

The technique used by us at Belfast was that of Fussell (1960) with a modified collection apparatus (Squance and Brown, 1965). Objections to the use of operated hens have been based on observations of physiological abnormality. This has been shown not to be the case by Richardson, Watts, Wilkinson and Dixon (1960), who have published a comparison of colostomised and normal hens and found no differences in nitrogen partition. In a similar experiment the present author compared colostomised and normal hens of the same age, fed a diet containing 17 per cent crude protein, and again no significant differences in partition were found. These results are shown in Table 1. The creatinine and ammonia levels are within the range of variability found in a number of determinations on control hens. In two cases disturbance of the water consumption pattern was observed with no apparent effects on nitrogen metabolism.

#### *Determination of True Digestibility*

The determination of true digestibility requires a reliable estimate of metabolic faecal nitrogen (MFN) output. This nitrogen fraction consists mainly of digestive enzymes and sloughed-off gut tissue.

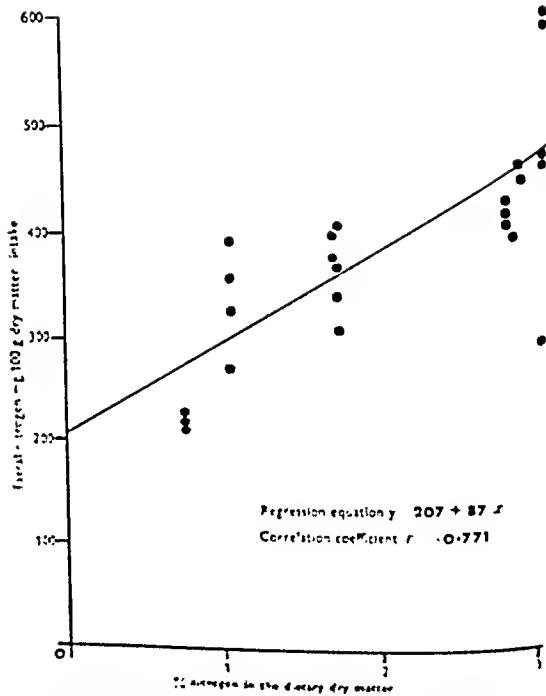


FIG. 1. Determination of MPN by extrapolation. Regression of faecal nitrogen - g/100 g (y) on percentage nitrogen in the diet dry matter (x). (Black points one (plus) trial period in one herd.)

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#### *Determination of True Digestibility*

The determination of true digestibility requires a reliable estimate of metabolic faecal nitrogen (MFN) output. This nitrogen fraction consists mainly of digestive enzymes and sloughed-off gut tissues

Direct determination of metabolic faecal nitrogen and endogenous urinary nitrogen were carried out using colostomised hens fed on a nitrogen-free diet based on that of Fisher and Johnson (1956). The diet was modified to include 12 per cent corn oil, and cellulose was excluded. Four colostomised hens were fed this diet for a 7-day pre-experimental period followed by a 5-day experimental period when urine and faeces were quantitatively collected. The results are presented in Table 2. These results compared well with a similar determination by Morimoto, Kubota, Ariyoshi and Hizikuro (1961).

TABLE 2

*Direct determination of metabolic faecal and endogenous urinary nitrogen on four hens fed a nitrogen free diet*

Hen No.	22	23	25	29	Mean values
Mean body weight (kg.)	1.845	1.980	1.700	2.045	—
Total MFN (mg.)	370.0	625.0	552.0	543.0	—
MFN/day (mg.)	92.5	125.0	110.0	108.6	109.3
MFN/100 g. DM intake/day	153.4	165.6	147.8	165.8	158.1
Total EUN (mg.)	543.6	495.4	617.0	747.0	—
EUN/day (mg.)	139.9	123.8	123.4	149.9	134.1
EUN/kg. body weight $\frac{2}{3}$ /day	88.6	74.2	82.9	87.4	83.3

We have also carried out an indirect method of determining metabolic faecal nitrogen, first used by Mitchell and Bert (1954), involving the feeding of a series of protein levels and at each level determining the faecal nitrogen. A regression of faecal nitrogen/100 g. dry matter consumed/day on the percentage nitrogen in the dietary dry matter is calculated (see Fig. 1), and the MFN is calculated as the faecal nitrogen at zero intake. The metabolic faecal nitrogen value of 116 mg. N/100 g. dry matter obtained from the regression and mean daily feed intake was significantly lower than that obtained by direct determination, viz. 158 mg./100 g. dry matter. This difference was also noted by Twombly and Meyer (1961) in the rat and it would suggest that the loss of endogenous nitrogen is reduced, or absorption of nitrogen from the gut enhanced under protein feeding conditions.

In applying the metabolic faecal nitrogen figure to calculations of true digestibility no account is taken of the effect of dietary protein *per se* on enzyme production. Nasset and Jin Soon Ju (1961), working with dogs and rats, showed that endogenous nitrogen secretions were four times the dietary nitrogen intake in dogs and six times the level in rats. Twombly and Meyer (1961) showed a definite increase in endogenous nitrogen production up to 15 per cent protein in the diet. This would suggest that there is an increase in enzyme production or tissue loss at higher intakes. Recently this work was confined by Snook and Meyer (1964), who examined activities of individual enzymes on a nitrogen-free diet and found increased activity when casein or egg protein were

fed. It must be remembered, however, that in all work using gut section analysis, no indication is given of the breakdown or re-absorption of the endogenous N. Thus much of this increased output could be re-absorbed.

One way to determine the exact effects of protein level on metabolic faecal nitrogen is to use labelled proteins. Work in this direction is being pursued by the present author using parenterally administered  $^{35}\text{S}$ -labelled methionine as a marker for endogenous proteins but considerable difficulties, of establishing equilibrium, are being encountered.

The metabolic faecal nitrogen previously determined by the direct method was used in the calculation of true digestibility in two experiments using practical laying diets.

In the first experiment the diets contained only maize and fish-meal as protein sources with sucrose added to increase the metabolisable

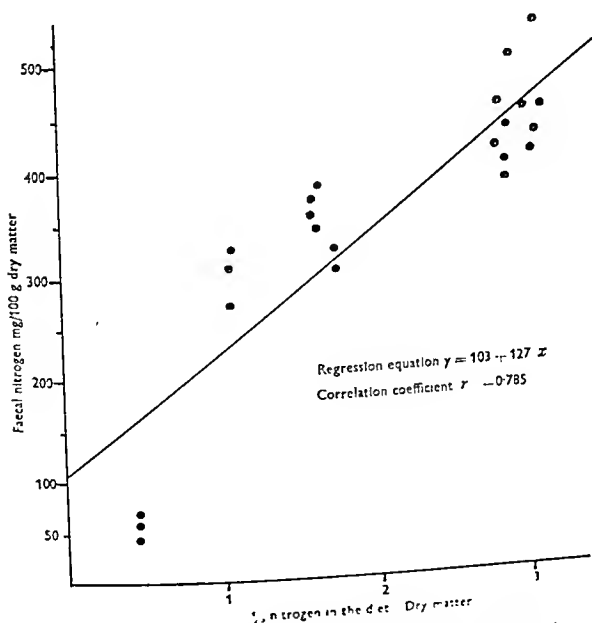


FIG. 1. Determination of MFN by extrapolation. Regression of Faecal N/100 g. D.M. (y) on percentage nitrogen in the diet dry matter (x). (Each point one 4-day trial period on one hen)

energy level to 2,929 kcal./kg. The low-energy diets were more conventional, being of mixed cereals with added fishmeal and had a metabolisable energy content of 2,533 kcal./kg. These diets were similar to those used by Brown, Waring and Squence (1965) to test the effects of calorie:protein ratio in egg production.

The determinations of true and apparent digestibility on these diets are given in Table 3. It can be seen that differences between diets were not significant.

TABLE 3

*Mean apparent and true digestibility and daily protein requirement of diets used in Experiment 1*

Diet	Apparent digestibility	±S.E. of mean*	True digestibility	±S.E. of mean	Calculated protein requirement (g./day)	Recorded daily intake of protein (g.)
High energy	17% C.P. 85.7†	—	89.0	—	—	—
	15% C.P. 86.3	—	91.0	—	13.3	14.1
	13% C.P. 82.4	±2.42	88.1	±0.62	13.2	13.2
Low energy	17% C.P. 82.0	—	86.0	—	12.3	18.0
	13% C.P. 81.2	—	85.7	—	11.9	15.7

\* Standard errors only quoted where 3 determinations made.

† All figures without standard error mean of 2 determinations.

The diets in the second experiment were from a laying trial designed to test the efficiency of egg production on a diet of 14 per cent protein with soyabean as the protein supplement. This diet was compared with one of 17 per cent crude protein containing 15 per cent of fishmeal. The basal soyabean diet was also supplemented with 0.09 per cent methionine and 0.2 per cent lysine alone and in combination. The determined metabolisable energy value of the soyabean diets was 2,810 kcal./kg. dry matter and of the fishmeal diet, 3,101 kcal./kg. dry matter. The results are presented in Table 4.

The true and apparent digestibilities in this experiment show no significant differences between treatments. The mean true digestibility value for the two experiments was 88.5 per cent, which compares well with the value of 80 per cent obtained by Morimota, Kuboto and Ariyoshi (1961) for maize-based Japanese diets and with that of MacDonald and Bose (1944) who obtained 85.6 per cent on mixed cereal/soya diets. Laerdal *et al.* (1957) obtained 88.7 per cent using male chicks fed a diet of maize and soyabean meal.

From the results of these experiments and published data it would seem that true nitrogen digestibility is independent of protein source. From results presented in Table 5, protein level does not influence protein digestibility over a wide range of protein levels. Practically, apparent digestibility is of more value than true digestibility as metabolic faecal nitrogen is part of the overall nitrogen loss to the bird and in the two experiments reported here apparent digestibility was 95 per



cent of true digestibility. Digestibility of protein is thus fairly constant both in practical and purified diets. The 10 per cent of undigested feed nitrogen is presumably in a form unaffected by digestive enzymes, or so occluded by fibrous material as to be unattacked by enzymes.

TABLE 4

*Mean apparent and true digestibility and daily protein requirement of diets used in Experiment 2*

Diet	Apparent digestibility	±S.E. of mean*	True digestibility	±S.E. of mean	Calculated protein requirement (g./day)	Recorded daily intake of protein (g.)
Fishmeal control	85.7	± 0.94	89.4	± 0.37	15.4	18.0
Soyabean control (SBC)	83.4	± 0.58	87.8	± 0.50	17.5	15.7
SBC+L-lysine	87.0	± 0.32	90.9	± 0.36	13.7	17.2
SBC+DL-methionine	85.6	± 0.22	90.3	± 0.52	12.4	14.7
SBC+L-lysine +DL-methionine	86.0	± 0.64	89.9	± 0.45	11.5	17.7

\* Each figure mean of 3 determinations.

TABLE 5

*Effect of protein level on protein digestibility in hens fed a purified diet (Fisher and Johnson, 1956) and supplementary protein as defatted whole egg. (Each figure 4-day trial on one hen)*

Protein level	5 per cent	10 per cent	15 per cent	20 per cent
True digestibility	88.0	90.6	88.2	89.9
	88.7	85.8	91.3	91.1
	—	85.2	90.3	98.7
	—	88.6	89.6	90.6
	89.3	87.6	90.6	85.2
	—	86.9	86.6	—
S.E. of mean	88.7	87.5	89.4	89.3
	0.36	0.80	0.69	1.06

### *Determination of Protein Requirement*

Consideration has been given to the possibility of using biological values to assess daily protein requirement for egg production by a factorial method. In calculating a factorial estimate of the protein requirement of laying hens from protein metabolism studies on a particular diet the main factors to be considered are the endogenous nitrogen losses, the requirements for egg production and body weight gain and the mean biological value and digestibility of the diet. From our work with the colostomised hen the endogenous losses of metabolic

faecal and endogenous urinary nitrogen are equivalent to 0.829 g. protein/kg. body weight. The egg production factor is the product of the mean egg weight, mean level of production and protein content of the egg which is 11.2 per cent. In the case of the light hybrid, the body weight changes have been ignored as they are small in relation to the whole laying period. For a simplified general equation as shown below we may assume a 60 g. egg and 75 per cent production. Using the mean biological value of 58 per cent determined for practical diets by us (Squance and Brown, 1965), we have calculated the requirement of protein for a 2 kg. hen to be 13.3 g./day. This is below the daily intake on all diets in Experiments 1 and 2 above (see Tables 3 and 4) and would suggest that the diets examined contained more protein than was required for the level of production.

However, when the biological value of each diet (Squance and Brown, 1965) is used, the intake of protein on the soyabean control diet was the only one below the calculated requirement (see Tables 3 and 4).

The simplified formula for the factorial method as used in this determination was as follows:

$$R_p = \frac{M \times W + (E_w \times E_p \times 0.112)}{BV \times D}$$

where  $R_p$  = Protein requirement (g./day)

$M$  = Maintenance requirement as protein (g./kg. body weight/day)

$W$  = Body weight (kg.)

$E_w$  = Mean egg weight (g.)

$E_p$  = No. of eggs/day

$BV$  = Biological value of diet (expressed as a fraction)

$D$  = Digestibility of diet (expressed as a fraction)

This simple formula will give a minimum requirement which is by no means complete. A full formula using all the factors referred to, including body weight change and feather loss, is given below:

$$R_p = \frac{(MFN \times F_e + EUN \times W^{\frac{1}{2}}) \pm W + (E_p \times E_w \times 0.112)}{BV \times D}$$

where  $MFN$  = Metabolic faecal nitrogen/g. diet.

$EUN$  = Endogenous urinary nitrogen/kg. body weight  $^{\frac{1}{2}}$ /day

$F_e$  = Dry matter intake (g./day)

$W^{\frac{1}{2}}$  = Metabolic body size

$\Delta W$  = Protein requirement for growth and feather replacement.

In this equation an account has been taken of the effect of dry matter intake on  $MFN$  output and  $EUN$  has been expressed in terms of metabolic size. Before a full estimate can be made the factors of growth and feather loss have to be ascertained. Information on this aspect of protein loss in high-producing hens is not readily available.

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AMINO ACID INTERACTIONS IN  
POULTRY NUTRITION

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IN ADDITION to specific amino acid inadequacies, the absence of an ideal dietary amino acid balance will also result in a retardation of growth. Though it may be possible to classify amino acid interactions into imbalances, antagonisms or toxicities it is not justifiable to infer that disparate mechanisms are involved. Further information on the nature of certain interactions is sought by studying the circumstances under which each amino acid can behave as the agent of interaction effects or as the target which must be supplied to counteract these effects.

*Introduction*

It is generally accepted that the efficiency with which a protein can be utilised depends upon how closely the proportion of the indispensable amino acids it provides corresponds to the needs of the animal. It also depends upon the ratio of indispensable to dispensable amino acids. Such a concept of amino acid balance is of critical importance in mammalian nutrition since there is essentially no storage of amino acids in the body.

The outlook until relatively recently in regard to the importance of amino acid balance in nutrition accommodated the assumption that any surplus of amino acids not used for protein synthesis exerted no adverse effects. This still appears to be true in general, but the observation that amino acid needs increase as the protein content of the diet is raised precludes its total validity. This effect of lack of balance upon requirements is currently an issue of very considerable importance. The concept of imbalance, on the other hand, has a more specific meaning and refers to those instances when a lack of balance causes a severe depression in growth rate. The situation can be exemplified by the observation of Salmon (1954) that the addition of gelatin to a low protein diet caused a depression in growth that was specifically prevented by tryptophan. Interactions of this type amongst amino

acids have been demonstrated on many occasions during the last decade.

It was proposed by Harper (1958) that such amino acid interactions could be divided into three categories: imbalances, where the effect of the added amino acid is reversed by supplementing the diet with the limiting amino acid; antagonisms, where the effect is reversed by an amino acid other than the limiting one; and toxic effects where no single amino acid supplementation reverses the growth depression. Although it is feasible to classify interactions in this way it is not justifiable to infer that disparate mechanisms are involved. More detailed information on individual instances of interaction is necessary before assuming an underlying cause of the phenomena.

There are two general mechanisms that have been proposed to account for these interactions. The first of these proposes that the effect of the added amino acid is to cause impaired utilisation of another. The presence of excess amino acid is thought to increase the activity of the mechanisms of disposal and concomitantly some of the limiting amino acid may be lost. Furthermore, if the growth rate of an animal is decreased a greater proportion of its dietary intake is diverted to maintenance which itself implies less efficient utilisation; but there is no clear evidence suggesting that impaired utilisation is the first cause of growth depression.

The second general mechanism proposed by Fisher and Shapiro (1961) implies that the depression in food intake which always results from the ingestion of an imbalanced diet is itself sufficient to account for the growth depression. It is possible that an elevated or deranged pattern of plasma amino acids might result in the reduced food intake via a hypothalamic mechanism.

### *Experimental*

The outlook taken in a programme of research at the University of Nottingham is to examine the detail of interaction between amino acids, particularly in terms of its scope. In the first instance the interaction between lysine and arginine was examined with the thought that subsequent studies would be designed with threonine and tryptophan, with leucine, isoleucine and valine, or with other groups. The approach was based on the concept of an agent amino acid invoking the response and a target amino acid which required also to be added to restore growth (Smith and Lewis, 1964a). The reversibility of the interaction was examined and also the scope of alternative agents or targets.

In the first instance it is possible to demonstrate the lysine-arginine interaction. The basal diet used in a series of experiments is described in Table 1. The arginine level is marginally adequate (see Lewis, Smith and Payne, 1963) whereas the lysine supply is sufficient. The addition of lysine to the basal diet (Table 2) results in a marked growth depression which is prevented if arginine is also added. An experiment

has been carried out (A12) using the same basal diet to test whether histidine can act as an alternative agent of interaction. The addition of histidine caused some depression in growth at the 6-week stage (Table 3), but it was much less severe than on adding lysine. If

TABLE 1

*Basal diet (A11-A14)*

(Composition expressed as percentage of dry matter except for protein which is expressed on an air-dry basis. The supplement contains the levels of amino acid stated: the figures for composition do not include the supplementary amounts)

Ingredients (per cent)		Composition (per cent)	
Maize meal	55	Protein (N×6·25)	21·6
Maize gluten meal	37	Arginine	0·79
Dried whey	3	Histidine	0·46
Supplement	5	Lysine	0·42
DL-methionine	0·1	Methionine + cystine	0·95
DL-tryptophan	0·2	Tryptophan	0·14
L-tyrosine	0·2		
L-lysine	0·6		
Glycine	0·3		

TABLE 2

*Liveweight and food conversion efficiency of birds fed diets supplemented with either lysine or arginine or both amino acids (A11)*

(Mean values for 6 replicates of 10 birds: groups of 15 birds placed in cages of day-old, reduced to 10 at 7 days and experimental diets given)

		Treatment	3 weeks	6 weeks
Liveweight (g.)	{	Basal	264	751
		+0·2 per cent arginine	267	738
		+0·6 per cent lysine	238	666
		+0·2 per cent arginine and 0·6 per cent lysine	271	759
		S.E.	± 5·1	± 12·0
Food conversion (g. food/g. weight gain)	{	Basal	1·76	2·25
		+0·2 per cent arginine	1·71	2·23
		+0·6 per cent lysine	1·83	2·39
		+0·2 per cent arginine and 0·6 per cent lysine	1·69	2·18
		S.E.	± 0·027	± 0·034

It is possible that the absence of any response in testing for tryptophan as an alternative target is accounted for by the relatively high tryptophan content of the basal diet in relation to arginine. A further basal diet was therefore prepared (Table 5) in which the tryptophan content was only 0.07 per cent. The addition of tryptophan resulted in a marked growth stimulation (Table 6) but supplementation with lysine did not depress growth. There was however a slightly unexpected depression on adding arginine alone or in the presence of tryptophan.

TABLE 3

*Liveweight of birds fed diets supplemented with arginine, lysine, histidine or arginine and histidine (A12)*

(Mean values in g. for 6 replicates of 10 birds)

Treatment	3 weeks	6 weeks
Basal	256	740
+0.2 per cent arginine	285	773
+0.3 per cent lysine	219	659
+0.15 per cent histidine	253	720
+arginine and histidine	273	766
+arginine and histidine	283	827
S.E.	±6.6	±20.9

TABLE 4

*Liveweight of birds fed diets supplemented with arginine, lysine, tryptophan or a mixture of three amino acids (A14)*

(Mean values in g. for 5 replicates of 8 birds)

Treatment	2 weeks	4 weeks
Basal	177	345
+0.2 per cent arginine	174	376
+0.6 per cent lysine	141	228
+0.2 per cent tryptophan	183	363
+arginine and lysine	162	306
+tryptophan and lysine	135	180
+arginine and tryptophan	177	323
+arginine, tryptophan and lysine	156	239

The basal diet was also used in a preliminary experiment (A16) to identify a tryptophan-threonine interaction (see Florentino and Pearson, 1962). The results in Table 7 show that tryptophan addition resulted in a growth stimulation but that the depression noted on adding threonine was not statistically significant. It is possible that in this case the tryptophan content is too low for an interaction effect to be demonstrated.

It is apparent from these observations that the interaction effects are not easily demonstrated. It has not been possible to show clearly any alternative agents to lysine or targets to arginine in this interaction: nor has it been possible to show the reversal of the process. It is either



possible that interactions of this type are of limited distribution or that they are only invoked when relative amino acid balance is subtly poised to produce a particular relationship.

TABLE 5

*Basal diet with a low tryptophan content (A15-16)*

*(Composition expressed as percentage of dry matter except for protein which is expressed on an air-dry basis. The supplement contains the levels of amino acid stated: the figures for composition do not include the supplementary amounts)*

Ingredients (per cent)		Composition (per cent)	
Maize meal	64	Protein (N $\times$ 6.25)	22.6
Maize gluten meal	20	Arginine	0.78
Zein	7	Lysine	0.34
Dried whey	3	Tryptophan	0.07
Supplement	6		
DL-methionine	0.1		
L-tyrosine	0.1		
L-lysine	0.6		
L-arginine	0.3		
Glycine	0.6		

TABLE 6

*Liveweight of birds fed varying amino acid supplements (A15)*  
*(Mean values in g. for 5 replicates of 8 birds)*

Treatment	2 weeks	3 weeks
Basal	132	163
+0.2 per cent arginine	129	103
+0.6 per cent lysine	133	167
+0.2 per cent tryptophan	180	295
+arginine and lysine	131	145
+tryptophan and lysine	185	305
+arginine and tryptophan	168	272
+arginine, tryptophan and lysine	186	314

TABLE 7

*Liveweight of birds fed a diet supplemented with tryptophan or threonine or a mixture of the two (A16)*  
*(Mean values in g. for 6 replicates of 6 birds)*

Treatment	2 weeks	3 weeks
Basal	159	236
+0.15 per cent tryptophan	182	291
+0.35 per cent threonine	158	223
+0.65 per cent threonine	157	222
+tryptophan and 0.35 per cent threonine	179	287
+tryptophan and 0.65 per cent threonine	177	284

It is also appropriate to comment briefly about another programme of research, impinging upon this issue, essentially carried out by Dr G. H. Smith, now at Leeds University. This work was done because of

the hypothesis that the extent to which an amino acid caused a deleterious effect when present in excess might be related to the ease with which it entered into metabolic pathways of catabolism and was removed from the metabolic pool. One may contrast the behaviour of lysine and arginine: the former is, in the mammal at least, relatively metabolically inert and is a potent agent of interaction, whereas the reverse is true in the case of arginine. A study of the pathways of catabolism of amino acids in the bird was therefore initiated. Generally

TABLE 8

*Oxygen uptake of acetone powders of chick and turkey liver*

(Incubation at 27°C. in presence of  $2 \times 10^{-4} M$   $CN^-$ : molarity of substrate, 0.02 M; pH, 8.0: activity expressed as  $\mu$  mole.  $O_2$  absorbed/g. powder/hr.)

	Whole liver	Nuclei and mitochondria	Supernatant	
			Fraction precipitated at pH 5.5	Remainder
<i>Turkey</i>				
Control	19.9	12.5	15.4	17.7
+ Lysine	27.5	15.6	23.7	26.2
<i>Chick</i>				
Control	10.3	7.0	17.0	6.9
+ Lysine	10.4	7.1	20.0	7.2

L-amino acid oxidases are of limited occurrence and low activity in mammalian systems, but there are indications that direct oxidative deamination may be more of significance in birds. Such an enzyme would, if of limited specificity and with an adaptive character, assist in elucidating the mechanism of the arginine-lysine interaction.

The oxidative properties of avian liver preparations were examined in the presence of added amino acids (Smith and Lewis, 1964b). The results in Table 8, using both turkey and chick liver preparations, show some increase in oxygen uptake upon adding lysine. Acetone powders were prepared from whole liver and various fractions obtained following centrifugation in isotonic sucrose. Cyanide was added to inhibit cytochrome oxidase activity. It was indicated by incubation in the presence of hydroxylamine (Table 9) that the process was probably not one involving transamination. From these observations it can at least be suggested that a mechanism which might account for some of the phenomena of amino acid imbalances, is an increase in oxidative catabolism in response to excess amino acids which carries with it the limiting amino acid. The possibility of an adaptive increase in activity of the L-amino acid oxidase in response to dietary intake has been demonstrated (Table 10). Insufficient results are available on the basis of which to decide whether these small differences are statistically significant.

Both these research programmes are continuing. Attempts are also being made to study the basic mechanism of the interaction by following plasma amino acid levels and the pattern of excreted nitrogen under defined circumstances of dietary amino acid balance and recorded

TABLE 9

*Oxygen uptake of the "pH 5.5" fraction of chick liver*

*(Incubation at 37°C in presence of  $2 \times 10^{-4} M CN^-$ : substrate, 0.03 M; pH, 8.0)*

Amino acid	No $NH_2OH$	Oxygen uptake ( $\mu$ mole/g./hr.) $+ 2 \times 10^{-3} M NH_2OH$	Relative rate by oxidase of Struck and Sizer (1960)
Control	18.3	17.4	—
Histidine	40.1	38.4	27
Arginine	39.2	—	51
Leucine	34.8	—	100
Lysine	29.9	29.7	78
Tryptophan	29.8	—	—
Glutamic acid	25.9	13.1	—
Phenylalanine	21.5	—	75
Methionine	18.7	—	48
Valine	18.6	—	1

TABLE 10

*Oxidase activity of chicks on different diets*

*(Mean oxygen uptake of acetone-dried "pH 5.5" fraction of liver in presence of 0.02 M substrate and  $2 \times 10^{-4} M CN^-$  at 37°C and pH 8.0: activity is expressed as  $\mu$  mole  $O_2$  absorbed/hr./g. powder: each group is of ten birds of 6 weeks of age)*

Groups receiving normal diet (0.9 per cent lysine)

	I	II
Control	14.1	12.1
+ 0.02 M-leucine	16.5	13.8
+ 0.02 M-lysine	14.3	14.1
Mean wt. of birds (g.)	760	720

Groups receiving diet with excess lysine (1.5 per cent)

	III	IV
Control	11.6	13.0
+ 0.02 M-leucine	16.4	14.2
+ 0.02 M-lysine	14.1	13.6
Mean wt. of birds (g.)	450	330

growth depression. That the agent amino acid might exert its effect by an influence of competition at alimentary or cellular absorption sites should also be considered.

A final point can be made on the application of these circumstances to conditions of poultry production. When all imbalances have been eliminated and a perfect dietary amino acid balance is achieved, the requirements for amino acids are minimal. Allowances that are recommended must include a graded margin to accommodate likely

deviations from an ideal balance. When the lysine level is optimal it is found that 0.8 per cent dietary arginine is adequate; for every 0.2 per cent increase in lysine above this it would seem the need for arginine goes up by 0.1 per cent.

It is only when basic biochemical interactions are understood that experimental nutritional observations can be clearly interpreted.

### *Acknowledgement*

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## AMINO ACID ALLOWANCES FOR LAYERS

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THE OPTIMAL amino acid allowances for layers have, in many instances, been determined by procedures involving the addition to a basal diet of one or more essential amino acids in graded amounts. Any measured response to an added test amino acid serves only to define the level at which a second amino acid becomes limiting.

In the trial reported, a sequential arrangement of amino acid supplementation was aimed at, with the final object of defining a balanced pattern of amino acids.

*Introduction*

Although several different procedures have been adopted to determine the optimal amino acid allowances for layers, there is still much indecision with respect to actual recommended allowances. Before commenting upon an experimental programme that has been conducted it is appropriate to discuss some of the procedures that are available.

In some instances a purely empirical approach has been adopted, namely to analyse a diet known to support a good level of production and to deem the amino acid content to be the recommended allowance. This approach is too rigid since it is impossible to seek improvement by altering the level of any one individual amino acid. Altering the proportions of the protein-containing ingredients will change the levels of all the amino acids in the diet.

Another widely adopted approach involves adding graded supplements of free amino acids to intact protein diets until a maximum level of production is attained. In this procedure it is essential to define carefully in the diet the level of amino acids other than the one specifically being examined. In principle the response to the test amino acid may be regarded only to serve to define the level at which a second amino acid becomes limiting.

A third procedure can be exemplified by a formula proposed by Combs (1960, 1961a) to calculate the requirement for methionine

from a consideration of the factors which can affect the needs for an amino acid. The formula proposed was:

$$\text{Percentage methionine} = \frac{C(0.05W + 6.2\Delta W + 5E)}{4537T(1.52W^{0.653} \pm 3.26\Delta W + 3.29E)}$$

where  $C$ =kcal. metabolisable energy per lb. diet,  $W$ =average body weight of hens (g.),  $\Delta W$ =average daily body weight change (g.),  $E$ =average weight of eggs per day (g.) and  $T$ =a temperature correction factor. The term  $C$  represents a factor known to regulate voluntary feed intake;  $W$  allows for a greater requirement in the heavier bird;  $\Delta W$  accommodates any liveweight gain; and  $T$  is a temperature correction factor to allow for the fact that the needs of the bird differ between warmer and colder periods. Requirements for the other amino acids were calculated from their ratio to methionine in whole egg protein. It has been considered that allowances derived in this way are successful to account for different utilisation of dietary energy supply.

A different approach has been adopted by Fisher (1961). The allowance is calculated by summarising together an assumed amount of amino acid needed for maintenance and the amount appearing in a 50 g. egg. Values were expressed as intake of amino acid in mg. per bird per day. Since dietary energy level can influence food intake and hence amino acid intake, dietary amino acid levels must be adjusted accordingly to meet intake requirements.

Studies on nutritional allowances for the chick have been relatively successful with an approach based upon the use of free amino acids. However, such studies have been less successful when applied to the allowances for the laying hen. Johnson and Fisher (1959) were able to devise a diet which could support egg production, but egg weight and body weight of the birds were not maintained. Adkins, Harper and Sunde (1961) found that their diets supported body weight but only a poor level of production. Since the amino acids in such diets must be supplied as free amino acids and not as proteins, dietary ingredients tend to be atypical and secondary effects can influence the results. Further, the cost of free amino acids imposes a limitation upon the magnitude or duration of a feeding trial.

A further approach to establishing amino acid allowances has been used based upon the radioactive isotope,  $S^{35}$ . Diets containing this isotope were fed to laying birds and its incorporation into the hen's body tissues and into the egg protein was measured. It was postulated that an amino acid deficient diet would increase catabolism of the hen's body protein, resulting in an increased deposition of  $S^{35}$  in the egg protein. A comparison of the amount of  $S^{35}$  appearing in the eggs from control and test diets should enable the allowance for the test amino acid to be derived. Unfortunately inaccuracies of the technique and poor food intake have as yet prevented any success in the use of this method.

*Materials and Methods*

When deriving amino acid allowances from theoretical considerations it is impossible to account simultaneously for all the factors which can influence the allowance. It may, therefore, be of advantage to adopt a direct approach in which dietary levels can be related to performance under specified conditions. Amino acid allowances are conventionally expressed as a percentage of the diet at a certain total level of protein. Proteins provide essential amino acids and the materials for the synthesis of the non-essential ones. A given protein level can be made up of different amino acid patterns depending on the

TABLE 1  
*Composition of basal diets*

	A	B	C
Maize meal	50	45	35
Ground wheat	35	34	37.5
Fishmeal (white)	2.5	2.5	2.5
Soyabean meal (50 per cent protein)	0	5	10
Fat (H.E.F.)	2.5	3.5	5
Limestone	5	5	5
Supplement	5	5	5
	—	—	—
	100	100	100
Protein content (per cent N $\times$ 6.25)	10.5	12.5	14.5

proportion of cereal to animal protein. However, when maize, wheat and soyabean are the main protein-bearing ingredients 12-13 per cent protein provides marginal amounts of essential amino acids. The problem of defining amino acid allowances could thus be solved by adequately supplementing such diets with free amino acids. The fact that the amino acid supply in such diets is in some instances nearly adequate, however, inhibits the responses to added amino acids. A group of three basal diets of 10.5, 12.5 and 14.5 per cent protein respectively have been used in a trial to establish amino acid allowances (Table 1). It was at least expected that at the lower protein level the essential amino acid supply would be inadequate. The composition of the supplement is given in Table 2. The purpose of this initial experiment was to attempt to define which amino acids were limiting in the basal diets and the sequence of limitation. In any trial where only one amino acid is added in graded amounts to a basal diet the response serves only to define the level at which a second amino acid becomes limiting. In this case, a sequential arrangement of amino acid supplementation was aimed at with the final object of defining a balanced pattern of amino acids. The amino acid composition of the basal diet was determined by analysis of the ingredients used (Table 3) and the values compared with a series of proposed allowances. These were derived by judicious selection from the data now available upon amino

**TABLE 2**  
*Composition of supplement*  
*(expressed as final concentration in the ration)*

Limestone	per cent	2.7
Dicalcic phosphate	"	1.2
Sodium ehloride	"	0.3
<hr/>		
Manganese	mg./kg.	60
Zinc	"	40
Iron	"	20
Copper	"	2
Iodine	"	1
Cobalt	"	0.2
<hr/>		
Vitamin A	i.u./kg.	5,000
Vitamin D <sub>3</sub>	"	1,250
Vitamin E	"	4
Menadione Na bisulphite	mg./kg.	8
Cboline chloride	"	300
Ca pantothenate	"	8
Riboflavin	"	4
Polic acid	"	1
Vitamin B <sub>12</sub>	"	0.01
Nicotinic acid	"	20
<hr/>		
B.H.T. (antioxidant)	per cent	0.0125
DL-methionine	"	0.10
Carophyll 10 (pigment)	mg./kg.	10

Wheat to 5 per cent (level of inclusion)

**TABLE 3**  
*Amino acid composition of basal diets*  
*(DL-methionine (0.1 per cent) also included in supplement)*

	Per cent diet			Proposed allowance
	A	B	C	
Lysine	0.38	0.53	0.69	0.55
Histidine	0.26	0.29	0.34	0.20
Methionine	0.25	0.26	0.26	0.28
Methionine + cystine	0.49	0.52	0.55	0.50
Tryptophan	0.07	0.10	0.12	0.15
Phenylalanine	0.47	0.56	0.66	0.50
Phenylalanine + tyrosine	0.85	1.01	1.16	0.85
Leucine	1.01	1.12	1.23	0.80
Isoleucine	0.41	0.50	0.60	0.55
Threonine	0.40	0.52	0.64	0.40
Valine	0.51	0.65	0.67	0.60

acid requirements (Table 4). On the basis of these values it was considered possible that lysine, tryptophan, isoleucine, valine, phenylalanine and threonine were limiting or marginal. Sixteen experimental treatments were thus devised (Table 5).



A group of 1,200 White Leghorn hybrids were purchased at day-old and reared intensively. For the first 8 weeks of life the chicks received a standard chick starter ration of 19 per cent protein and 2,800 kcal. metabolisable energy per kg. From this stage until the trial commenced the birds received a diet of 16 per cent protein and 2,600 kcal.

TABLE 4  
*Essential amino acid requirement of the laying hen*  
(expressed as percentage diet)

	NRC (1960)	Fisher (1958)	Combs (1961b)	Proposed allowances
Lysine	0.50	0.55	0.60	0.55
Histidine	—	0.20	0.21	0.20
Methionine	0.28	0.26	0.28	0.28
Methionine + cystine	0.53	0.44	0.49	0.50
Tryptophan	0.15	0.13	0.15	0.15
Phenylalanine	—	0.46	0.50	0.50
Phenylalanine + tyrosine	—	0.79	0.87	0.85
Leucine	1.20	0.75	0.83	0.80
Isoleucine	0.50	0.55	0.60	0.55
Threonine	0.40	0.40	0.44	0.40
Valine	—	0.59	0.65	0.60

TABLE 5  
*Experimental treatments*

1. Basal diet A (10.5 per cent protein)
2. Basal diet + 0.1 per cent lysine
3. Basal diet + 0.05 per cent tryptophan
4. Basal diet + 0.1 per cent lysine tryptophan
5. Basal diet + 0.1 per cent lysine + 0.05 per cent tryptophan + 0.05 per cent isoleucine
6. Basal diet + 0.1 per cent lysine + 0.05 per cent tryptophan + 0.05 per cent valine
7. Basal diet + 0.1 per cent lysine + 0.05 per cent tryptophan + 0.05 per cent isoleucine + 0.05 per cent valine
8. Basal diet + 0.1 per cent lysine + 0.05 per cent tryptophan + 0.05 per cent isoleucine + 0.05 per cent valine + 0.025 per cent phenylalanine + 0.025 per cent threonine
9. Basal diet B (12.5 per cent protein)
10. Basal diet + 0.05 per cent lysine
11. Basal diet + 0.025 per cent tryptophan
12. Basal diet + 0.05 per cent isoleucine
13. Basal diet + 0.05 per cent lysine + 0.025 per cent tryptophan + 0.05 per cent isoleucine
14. Basal diet C (14.5 per cent protein)
15. Basal diet + 0.05 per cent lysine
16. Basal diet + 0.025 per cent tryptophan

metabolisable energy per kg. At 16 weeks of age (September 1963), the birds were placed in the laying cages, three birds to each 17-in. laying cage. There were four replicate blocks each containing all the treatments arranged in such a way as to reduce any effect due to proximity to the door or height from the floor.

The trial was continued until the birds were 500 days of age. Records were kept of egg numbers, egg weight, food intake, body

weight and mortality. Representative eggs were periodically analysed for albumen quality measured as Haugh units.

### *Experimental Results and Discussion*

No significant differences were observed in mortality or in internal albumen quality measured as Haugh units. The results obtained during the experimental period are summarised in Table 6. There was a significant increase in egg weight between the 10.5 and 12.5 per cent protein diets, but not between the 12.5 and 14.5 per cent protein diets. Supplementation of the 10.5 per cent protein diet gave responses but only in rations 7 and 8 were these significant. It seems, therefore, that the 12.5 per cent protein diet provided sufficient amino acids to maintain egg weight.

There was a significant increase in egg production with increasing dietary protein level. Supplements to the 10.5 per cent protein diet gave significant increases, but production did not reach that of the 12.5 per cent protein diet. A lysine supplement to the 12.5 per cent protein diet gave an insignificant decrease, but tryptophan and isoleucine supplements resulted in significant increases and a mixture of all three amino acids gave an encouraging response to a level which equalled that of the 14.5 per cent protein diet. At the 14.5 per cent protein level, a lysine supplement gave a marked depression, but a tryptophan supplement gave a good response and this diet supported excellent egg production.

The responses shown to lysine supplementation merit further attention. At the 10.5 per cent protein level there was a marked increase showing lysine to be limiting whereas at the 12.5 per cent protein level there was no response, and at the 14.5 per cent protein level there was a marked depression indicating the lysine allowance to have been extended. It would in fact appear that in the 10.5 per cent protein diet, lysine was the first limiting amino acid, whereas in the 12.5 per cent protein diet it was isoleucine and in the 14.5 per cent protein diet, tryptophan.

A comparison of rations 5, 7 and 8 with ration 6 suggests that the inclusion of isoleucine reduces the response to added amino acids. This effect may be attributed to an imbalance involving isoleucine. At the 12.5 per cent protein level, an isoleucine supplement gave a marked response. Similar effects were noted by Bray (1964) who found an imbalance at the 9 per cent protein level, which could be corrected by supplements of other amino acids.

March and Bieley (1963) have suggested that amino acid balance could modify egg weight and that balance of amino acids is more critical for egg weight than for egg production. Results here suggest that amino acid imbalance affected production rather than egg weight. Johnson and Fisher (1959) showed that a 10.4 per cent protein diet could maintain production, but not egg weight and suggested a lack of

TABLE 6  
*Summary of results during the experimental period*

Treatment	Egg production (hen day) 12 month mean	Total eggs		Egg weight (g.) 12 month mean	g. food/ bird/ day	kg. food/ doz. eggs	g. food/ g. egg
		500 days	52 weeks				
1. 11.5 per cent protein	48.3	162	176	54.4	114.5	2.84	4.35
2. 11.5 per cent L	55.6	187	203	55.7	117.0	2.53	3.78
3. 10.5 per cent T	50.8	171	185	55.2	116.0	2.74	4.14
4. 11.5 per cent L T	53.5	180	195	55.6	116.0	2.60	3.90
5. 11.5 per cent L T I	55.1	185	201	55.7	115.0	2.50	3.75
6. 11.5 per cent L T V	58.1	195	212	55.9	115.5	2.39	3.56
7. 11.5 per cent L T I V	53.5	180	195	56.2	114.5	2.57	3.81
8. 10.5 per cent L T I V Ph Th	54.7	184	200	56.1	115.5	2.53	3.16
9. 12.5 per cent protein	60.0	202	220	56.8	115.0	2.30	3.37
10. 12.5 per cent L	59.2	199	216	56.8	115.5	2.34	3.43
11. 12.5 per cent T	62.7	211	229	56.6	114.5	2.19	3.23
12. 12.5 per cent I	63.2	212	231	56.8	114.5	2.17	3.19
13. 12.5 per cent L T I	67.0	225	245	57.3	115.0	2.06	2.99
14. 14.5 per cent protein	67.7	227	250	57.4	114.0	2.02	2.93
15. 14.5 per cent L	64.6	218	236	57.5	114.0	2.12	3.07
16. 14.5 per cent T	71.8	241	262	57.5	114.5	1.88	2.77

non-essential nitrogen was the cause of this. It is possible therefore that the balance of amino acids exerts an effect on egg weight when there is a shortage of non-essential nitrogen. In the case reported here, there was no such lack and effects of imbalance manifested themselves in egg production rather than egg weight.

Food intake data (Table 5) show no differences expressed as g. per bird per day. Some reports have appeared indicating an increased food intake to compensate for low protein levels. In this instance, however, the homeostatic energy regulation seems to have been the dominating factor. Food intake expressed as g. per bird per day showed no significant differences. There was no tendency to increase food intake to compensate for amino acid inadequacies.

Although one diet supported an excellent level of production (ration 16) it cannot be assumed that this diet provided the optimal amino acid allowances. Further studies are being conducted to establish limiting amino acids and to define optimal levels of supplementation. The aim will be to improve production through amino acid balance. In particular the relative levels of tryptophan and threonine and of isoleucine, leucine and valine will be more closely investigated. It is possible that antagonistic relationships between these amino acids exist in the laying hen.

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## BIOASSAY OF AMINO ACIDS

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IN ANY assessment of amino acid availability that involves the measurement of the rate of growth of chicks in relation to graded inclusions of standard amino acid and test protein, all responses to the test protein other than those due to its contribution of the amino acid being assayed must be eliminated. In this respect it is essential to avoid the differentials in dietary protein levels that generally result from the addition of graded levels of test protein. Furthermore, conditions of relative amino acid imbalance in basal diets and the varied changes imposed upon these by adding test protein will only serve to distort the response of the chick to the amino acid being assayed. Failure to eliminate these so-called protein effects considerably reduces the value of assay procedures so far reported.

In this light an experimental programme has been carried out to establish for the chick an optimum balance of amino acids in the dietary protein and to create a situation in which amino acid and test protein supplementation can be effected without invoking the so-called protein effect.

*Introduction*

The nutritive value of a protein is determined not only by its amino acid content but also by the availability to the animal of the individual amino acids. Despite the considerable attention that is now given to the amino acid composition of the proteins of poultry diets, it is seldom possible to state the proportions of the various amino acids which are available. Since processing and storage methods markedly influence amino acid availability it is particularly important to be able to carry out determinations on the types of diet that are fed.

Methionine and lysine are the two dietary amino acids most likely to impose a limitation upon the growth performance of poultry. It is therefore necessary to devise procedures to establish the biological availability of these amino acids. Carpenter (1960) has developed a chemical test that measures the extent to which the  $\omega$ -amino group of lysine is not involved in a chemical bond. This seems to produce a

value that is related to the availability of the amino acid but it does not take into account the other factors which can also modify availability. The results obtained by this technique ought to be regarded as maximum values. Several bioassay procedures have been proposed but in no instance has all the requirements for a satisfactory bioassay technique been met.

When information is sought regarding the availability of a nutrient it is essential that the determination be carried out using the species for which the knowledge is required. The full requirements for growth of the species must be known. The response measured in the assay must be a function only of the nutrient that is being assayed; no partial replacements, complementary effects or antagonistic responses to other factors can be acknowledged. The assay should be applicable over a wide range. The response of the test animals must be linearly related to the supplementary materials added, or to some function of such doses. The responses to graded doses of test and standard materials must show a statistically satisfactory relationship in terms of the parallel line or slope-ratio analysis. In this work the former is advocated essentially since the slope-ratio method normally adopted is not based upon a logarithmic dose assessment. A constant response is essential upon repeated assay and all secondary limiting factors must be eliminated from the working range of the assay. In the present work two aspects have received particular attention: the elimination of the so-called "protein effect", and the establishment of "maximum growth".

In any assessment of amino acid availability that involves the measurement of the rate of growth of chicks in relation to graded inclusions of standard amino acids and test protein, all responses to the test protein other than those due to its contribution of the amino acid being assayed must be eliminated. In this respect, it is essential to avoid the differentials in dietary protein levels that generally result from the addition of graded levels of test protein. Furthermore, conditions of relative amino acid imbalance in basal diets, and the varied changes imposed on these by adding test protein will only serve to distort the response of the chicks to the amino acid being assayed. Failure to eliminate these so-called protein effects considerably reduce the value of assay procedures. The assay procedure described here eliminates these effects by maintaining in all diets, equal protein levels, consistent amino acid balance and an equivalent contribution in terms of metabolisable energy.

### *Experimental*

In any bioassay procedure based upon growth it is important to ensure that a good overall nutrient balance is maintained. A poor amino acid balance will adversely affect utilisation just as will incomplete digestion and absorption. This would result in a false assessment

of low availability. It is, therefore, desirable to establish "maximum growth" and to maintain this standard with the higher levels of test supplementation even if only to demonstrate that no secondary factors are imposing a limitation upon growth.

In an attempt to establish a basal diet that accommodated good chick growth several mixtures were prepared based upon maize and sesame meal (3,250 kcal. metabolisable energy per kg.). Crystalline amino acids were added in each case to achieve a final level equivalent to 20 per cent protein (per cent N $\times$ 6.25). When the diets were

TABLE 1

*Effect of amino acid supplementation of maize-sesame meal diet (20 per cent protein, 3,250 kcal. metabolisable energy per kg., form of diets and nature of supplement as in Table 2)*

	Liveweight gain (g./day) 14-21 days	Per cent dietary nitro- gen retained (21 days old)	Food conversion ratio
Amino acids adjusted to NRC (1960)	16.0	50	2.01
Amino acids adjusted to 120 per cent NRC (1960)	18.5	58	1.84
Amino acids adjusted to Dean & Scott (1962)	21.0	63	1.71
Maize-sesame meal diet (+0.8 per cent lysine)	22.5	65	1.64

prepared in such a way that the recommendations of the National Research Council (1960) were only just met (see Table 1), the growth rate of the chicks within the period of 2 to 3 weeks was equivalent to 16 g. per day, but when the recommended levels were all increased by 20 per cent the total protein still remaining at 20 per cent of the diet, the rate of growth was 18.5 g. per day. In another instance the amino acid pattern was adjusted to that of Dean and Scott (1962) and the birds grew at a rate of 21 g. per day: a diet based solely upon the maize-sesame meal mixture with 0.8 per cent added L-lysine supported a growth rate equivalent to 22.5 g. per day. In all the diets used in the bioassay procedure developed, this final pattern of amino acid balance was maintained.

The essential nature of the bioassay procedure that has been devised is that various supplements are made to a 16 per cent protein basal diet, including both standard levels of lysine or protein sources, in each case to a stage equivalent to a 20 per cent protein basal diet. The composition of the basal diets is given in Table 2. The 16 per cent protein basal diet can be made equivalent to 20 per cent protein by the addition of 4 per cent protein. In preliminary experiments (Table 3) it was shown that a similar response was recorded when sesame meal or an amino acid mixture simulating sesame meal was used to bridge the

gap. In the actual bioassays the 16 per cent protein basal diet is made equivalent to 20 per cent protein by the addition of graded amounts of test protein and quantities of amino acids calculated to maintain the 20 per cent protein pattern. The standard series within the actual bioassays was also prepared from the same 16 per cent protein basal diet

TABLE 2

*Composition of basal diets (per cent)*

(Sesame meal of 38 per cent protein: the maize-fat proportions can be adjusted to give a constant energy level: the supplement provided in mg. per kg. diet, limestone 20,000, dicalcic phosphate 17,000, NaCl 3,000, Mn 80, Zn 50, Fe 20, Cu 2, Mo 2, I 1.0, Se 1.0, Co 0.2, Vit. A 6,000 i.u., Vit. E 5 i.u., Vit. K 9, choline chloride 800, pantothenic acid 10, riboflavin 5, folic acid 1.5, Vit. B<sub>12</sub> 0.02, nicotinic acid 25, procaine penicillin 25, amprolium 125, B.H.T. 125)

	A	B	C	D
Sesame meal	31	37	43	48
Maize meal	57	51	45	40
Fat	5	5	5	5
Dried whey powder	2	2	2	2
Supplement	5	5	5	5
Crude protein (N×6.25)	16	18	20	22

TABLE 3

*Effect of amino acid supplementation of maize-sesame meal basal diets*

(Details of composition as in Table 2, EAAS=essential amino acid mixture to bridge a gap, GA=glutamic acid, each result represents the means of 6 groups of 10 birds)

Treatments	Liveweight gain (g./day) 14-21 days	Food conversion ratio	Percentage of dietary nitrogen retained
1. Basal A	18.8	2.03	58.8
2. Basal B	19.5	1.91	60.4
3. Basal C	21.9	1.64	65.4
4. Basal D	22.3	1.66	65.2
5. 1+EAAS+GA=3	21.3	1.70	64.5
6. 3+5 per cent fat	21.5	1.69	62.8
7. 5+5 per cent fat	21.7	1.70	64.3
8. 3+5 per cent dried whey	21.1	1.70	65.0
9. 5+5 per cent dried whey	21.5	1.68	63.5

and in this case graded amounts of lysine were added together with quantities of the essential amino acids necessary to achieve equivalence with the 20 per cent protein basal diet plus an amount of glutamic acid to bring it actually to the 20 per cent protein level. Since all the amino acids and test protein supplementations are made at the expense of maize, slight adjustments are made in the maize-fat proportions. In this way a constant metabolisable energy level is maintained.

The standard diets are prepared by supplementing the basal diet



(16 per cent protein plus amino acid mixture) with 0.05 per cent, 0.1 per cent, 0.2 per cent and 0.4 per cent L-lysine hydrochloride. It is not advisable to use higher levels of lysine supplementation since it would jeopardise conditions of similarity with test levels essential for the parallel-line assay technique. For proteins of low lysine content e.g. groundnut meal, it may be necessary to eliminate the 0.4 per cent lysine level. The test protein supplementary levels are fixed by their protein and amino acid contents since the aim is to formulate all diets at the 20 per cent protein level. The total amino acid content of test proteins must be known in order to adjust the final amino acid pattern in each diet to that of the 20 per cent protein basal diet. Thus all test proteins are added in such a way as to supply less than 1 per cent, 2 per cent and 4 per cent protein respectively to the 16 per cent protein basal diet. This would raise the protein levels of the experimental diets to just under 17 per cent, 18 per cent and 20 per cent respectively. All diets are then made equivalent to the 20 per cent protein diet by the addition of calculated amounts of amino acids necessary to bridge the gap. These points are best illustrated by reference to an assay of fishmeal (FM 2).

The amino acid composition of the 16 per cent basal diet used for the assay is shown in Table 4. The difference between this and the composition of the 20 per cent protein basal diet is then made up as shown. For the standard series, the 16 per cent protein diet (with added essential amino acids to be equivalent to the 20 per cent protein diet) was supplemented with 0.1 per cent, 0.2 per cent and 0.4 per cent L-lysine HCl. Glutamic acid was added to make all diets equivalent in terms of nitrogen (equivalent to 20 per cent  $N \times 6.25$ ). The test protein FM 2 contained 72 per cent crude protein and the test series was prepared containing 1.25 per cent, 2.50 per cent and 5.0 per cent FM 2. The manner of supplementation with amino acids to a consistent protein level and amino acid balance is exemplified in Table 5. In all diets the maize-fat proportions are regulated (by using two pre-mixtures) to maintain a constant energy level and the appropriate quantities of glutamic acid are added to maintain a constant nitrogen content.

The full experimental details have been described by Uwaegbute (1964) and some of the results already obtained are summarised in Tables 6-9. The available lysine contents of the protein concentrates have been expressed as g. available lysine/16 g. N. The response parameters measured have been compared in terms of the precision of the estimate obtained with each of them. In this context, the width of the fiducial interval has been expressed as the mean percentage difference of the upper and lower fiducial limits from the quoted relative potency. It is clear from the tables that the narrowest fiducial intervals, and hence the most reliable estimates of potencies are those obtained from the nitrogen retention data. The explanations for this observation are both statistical and fundamental in origin. Fundamentally it is feasible to

suggest that the extent of body nitrogen retention is more closely related to the fate of ingested proteins and amino acids than liveweight gain and feed efficiency, since any increase in body liveweight is a function in which dietary factors other than proteins and amino acids

TABLE 4

*Composition of the adjusted basal diet*

(The 16 per cent protein basal diet was adjusted to accommodate up to 4 per cent amino acid mixture of test protein: the amino acid mixture used contained a contribution of glutamic acid equal to 1.7 per cent of the diet: the metabolisable energy value was equivalent to 3,290 kcal. per kg.)

	Basal diet (percentages)		Amino acid supplementation (percentage)	
	Sesame meal	31	Maize-fat	58
	Dried whey	2	Supplement	5
	Amino acid or protein	4	(minerals and vitamins)	
	20 per cent protein diet	16 per cent protein diet	Difference	Addition (g.) to 20 lb. diet
Arginine	1.62	1.26	0.36	32.8
Lysine	0.59	0.49	0.10	9.4
Histidine	0.42	0.34	0.08	7.3
Methionine + cystine	0.84	0.67	0.17	15.3
Tryptophan	0.30	0.23	0.07	5.9
Glycine	1.62	1.21	0.41	36.4
Phenylalanine + tyrosine	1.96	1.57	0.39	35.3
Leucine	1.58	1.35	0.23	20.4
Isoleucine	1.87	1.70	0.17	16.1
Threonine	0.88	0.76	0.12	10.9
Valine	1.08	0.85	0.23	20.2

TABLE 5

*Amino acid contribution of test protein (FM 2)*

(The diet containing 5.0 per cent FM 2 also contains 31 per cent sesame meal, 56.2 per cent of the maize-fat mixture and 0.8 per cent amino acid mixture)

	16 per cent protein diet per cent	5.00 per cent FM 2 per cent	Total per cent	Diff. from 20 per cent protein diet per cent	Amount added in 20 lb. diet g.
Arginine	1.24	0.26	1.50	0.12	11.1
Lysine	0.47	0.31	0.78	0.11	10.4
Methionine + cystine	0.66	0.16	0.82	0.03	2.4
Tryptophan	0.23	0.04	0.27	0.03	3.0
Phenylalanine + tyrosine	1.54	0.28	1.82	0.14	12.6
Threonine	0.74	0.12	0.86	0.02	2.0
Valine	0.84	0.19	1.03	0.05	4.6

are implicated. The greater precision of relative potency estimated from nitrogen retention data over liveweight gain data arise also from the greater replication in time which occurs when nitrogen retention is measured. The comparable number of replicates for liveweight gain and nitrogen retention are 4 and 12 respectively. It is proposed to examine this problem further to see whether the superiority of nitrogen retention over the other response parameters is still maintained when the number of treatment replications is the same for all response parameters.

TABLE 6

*Availability of lysine in fishmeals*

(Values indicated by the symbol  $\pm$  refer to the range of the fiducial limits for  $P, 0.05$ ; the materials assayed were withdrawn from the samples held by the A.R.C. Protein Quality Group)

Response parameter	FM 2 Available lysine g./16 g. N	FM 7 Available lysine g./16 g. N	FM 20 Available lysine g./16 g. N
Weight gain			
7-14 days	6.8 $\pm$ 8.1 per cent	6.4 $\pm$ 10.2 per cent	6.3 $\pm$ 11.8 per cent
Food efficiency			
7-14 days	7.2 $\pm$ 16.7 per cent	6.6 $\pm$ 15.8 per cent	6.7 $\pm$ 16.4 per cent
Weight gain			
14-21 days	7.0 $\pm$ 16.4 per cent	6.0 $\pm$ 12.5 per cent	6.2 $\pm$ 13.7 per cent
Feed efficiency			
14-21 days	7.5 $\pm$ 18.0 per cent	6.7 $\pm$ 18.6 per cent	6.3 $\pm$ 19.0 per cent
Nitrogen retention			
19-21 days	6.8 $\pm$ 11.0 per cent	6.4 $\pm$ 10.1 per cent	6.4 $\pm$ 10.1 per cent
Chemical assay	6.0	5.5	4.6

TABLE 7

*Availability of lysine in meat meals*

(Values indicated by the symbol  $\pm$  refer to the range of the fiducial limits for  $P, 0.05$ ; the materials assayed were withdrawn from the samples held by the A.R.C. Protein Quality Group)

Response parameter	MM 19 Available lysine g./16 g. N	MM 23 Available lysine g./16 g. N	MM 28 Available lysine g./16 g. N
Weight gain			
7-14 days	2.7 $\pm$ 42.4 per cent	3.5 $\pm$ 38.5 per cent	3.8 $\pm$ 21.5 per cent
Food efficiency			
7-14 days	2.4 $\pm$ 41.6 per cent	4.0 $\pm$ 17.5 per cent	3.0 $\pm$ 36.5 per cent
Weight gain			
14-21 days	3.5 $\pm$ 28.5 per cent	3.6 $\pm$ 13.8 per cent	3.3 $\pm$ 13.6 per cent
Food efficiency			
14-21 days	2.9 $\pm$ 18.9 per cent	—	3.7 $\pm$ 27.0 per cent
Nitrogen retention			
19-21 days	3.3 $\pm$ 7.6 per cent	3.5 $\pm$ 8.6 per cent	3.5 $\pm$ 5.7 per cent
Chemical assay	3.2	4.4	4.4

It can also be seen from the summary tables (6-9) of results that available lysine content computed from food efficiency data is generally greater than that computed from liveweight gain data in the same period. This is in agreement with other observations (Guttridge, 1962;

TABLE 8

*Availability of lysine in soyabean meals*

(Values indicated by the symbol  $\pm$  refer to the range of the fiducial limits for  $P, 0.05$ ; the materials assayed were withdrawn from the samples held by the A.R.C. Protein Quality Group)

Response parameter	SB 9 Available lysine g./16 g. N	SB 19 Available lysine g./16 g. N	SB 21 Available lysine g./16 g. N
Weight gain			
7-14 days	3.4 $\pm$ 27.9 per cent	3.2 $\pm$ 17.2 per cent	3.1 $\pm$ 14.5 per cent
Food efficiency			
7-14 days	3.9 $\pm$ 29.4 per cent	4.0 $\pm$ 28.7 per cent	3.5 $\pm$ 28.5 per cent
Weight gain			
14-21 days	3.4 $\pm$ 17.6 per cent	— —	— —
Food efficiency			
14-21 days	3.4 $\pm$ 27.9 per cent	3.5 $\pm$ 27.1 per cent	3.3 $\pm$ 27.2 per cent
Nitrogen retention			
19-21 days	3.4 $\pm$ 10.2 per cent	3.3 $\pm$ 9.1 per cent	3.3 $\pm$ 10.6 per cent
Chemical assay	4.1	3.6	2.7

TABLE 9

*Availability of lysine in groundnut meals*

(Values indicated by the symbol  $\pm$  refer to the range of the fiducial limits for  $P, 0.05$ ; the materials assayed were withdrawn from the samples held by the A.R.C. Protein Quality Group)

Response parameter	GN 12 Available lysine g./16 g. N	GN 18 Available lysine g./16 g. N	GN 19 Available lysine g./16 g. N
Weight gain			
7-14 days	2.2 $\pm$ 45.4 per cent	2.4 $\pm$ 54.1 per cent	3.6 $\pm$ 44.4 per cent
Food efficiency			
7-14 days	2.7 $\pm$ 37.0 per cent	2.9 $\pm$ 34.4 per cent	4.1 $\pm$ 31.6 per cent
Weight gain			
14-21 days	3.0 $\pm$ 35.0 per cent	— —	— —
Food efficiency			
14-21 days	— —	— —	— —
Nitrogen retention			
19-21 days	3.1 $\pm$ 9.7 per cent	3.2 $\pm$ 7.8 per cent	3.4 $\pm$ 7.3 per cent
Chemical assay	2.7	2.9	3.2

Carpenter, March, Milner and Campbell, 1963) and it is suggested that expressing growth as a function of food intake has the effect of reducing the magnitude of the differences between different dietary intakes and the growth responses they produce. This is reflected in an apparent higher relative potency when this is measured by the differences between the mean responses to the standard and test diets.

In general, the results show close agreement (in the ranking order of the protein concentrates) between the chemical and biological procedures. For the fishmeals available lysine content determined chemically was less than the values determined biologically. This is understandable because the chemical procedure measures only those lysine molecules containing free and reactive amino groups, with the assumption that only these are available to the animal. For the meat meals, although the chemical procedure yielded the same ranking order as the biological tests, the chemical values were higher than those

TABLE 10

*Chick bioassay of lysine (routine procedure)*

*(Amino acid contribution of the test protein; the column "ratio" refers to the relative content of the particular amino acid in the test protein and the sesame meal it replaces)*

	Ratio	12.255 per cent sesame meal per cent	6.5359 per cent FM 7 per cent	Difference per cent	Amount added in 20-lb. diet g.
Arginine	1.40	0.453	0.339	0.114	10.4
Lysine	—	0.145	0.400	0.145	13.2
Glycine	1.75	0.478	0.447	0.031	2.8
Threonine	1.89	0.196	0.196	—	—
Leucine	1.69	0.355	0.320	0.035	3.2
Valine	1.57	0.288	0.242	0.046	4.2

biologically determined especially for MM 23 and MM 28. It is probable that these meals contain significant amounts of bone, tendon and skin collagens which are known to contain significant quantities of hydroxylysine—which is nutritionally worthless, but measured all the same as available lysine by the chemical procedure. It is not yet necessary to question the assumption that lysine chemically bound is never biologically available.

The soyabean meals showed approximately the same available lysine content by the biological tests, but the chemical procedure gave values which were higher than the biologically determined values. It is possible that our recovery tests which yielded a correction factor of 1.20 may have overestimated the destruction of DNP-lysine during the acid hydrolysis stage. The indications, however, are that such a destruction was probably underestimated by the factor of 1.20. It is more probable that since the presence of any growth depressing agents in such meals cannot be detected by the chemical procedure, chemical values in such cases will be higher than the biologically determined values. For the groundnut meals, the relative ranking order of their available lysine content was similar from both the chemical and biological procedures. The chemical values were also in each case less than the biologically determined values.

A modification of this procedure is now being devised which is far less demanding upon a supply of free amino acids. The principle of the change is that the concept is based upon the 20 per cent protein diet rather than the 16 per cent protein diet. The standard series merely involves supplementation with graded amounts of lysine. In the case of the test series a quantity of sesame meal is withdrawn to allow the test protein to be introduced. Sufficient is withdrawn, however, to avoid a surplus of any amino acid. The level of withdrawal is defined by the amino acid present in the test protein in the greatest relative excess over sesame meal (Table 10). Any inadequacies of other amino acids resulting from this are made up by adding free amino acid. All these calculations are carried out on the basis of nitrogen.

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# CURRENT VIEWS ON THE ROLE OF THE GUT FLORA IN NUTRITION OF THE CHICKEN

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## *Synopsis*

MORPHOLOGICAL differences between germ-free and conventional chickens, and their possible significance in the absorption of nutrients, are discussed.

Nutritional studies with germ-free chickens are reviewed, and the following conclusions suggested. The gut flora has little effect on pancreatic enzymes. Germ-free birds have higher liver stores of vitamin A, and do not synthesise vitamin B<sub>12</sub> in the gut. Volatile fatty acids in chicken blood are not of microbial origin. Absence of a microflora leads to elevated levels of cholesterol in the plasma. Growth of young chicks may be depressed by some components of the intestinal flora.

The bacteriological aspects of studies on chicks with a defined gut flora are briefly discussed.

## *Introduction*

The belief that the microflora of the alimentary canal has an important influence on nutritional processes in the chicken has, until recently, been largely based on indirect evidence (cf. review by Coates, 1961). Modern techniques of gnotobiology allow a more direct approach to the problem, since it is now relatively easy to maintain birds in a "germ-free" state, that is, devoid of contamination with any demonstrable living micro-organism. Comparisons between germ-free and conventional chickens can indicate processes of digestion and metabolism that are influenced by the gut flora, and the components of the flora responsible for any particular effect may be detected by means of studies in birds contaminated with known organisms. However, some difficulty arises in interpreting results of such comparisons, since there are marked morphological and physiological differences between the germ-free animal and its mono- or poly-contaminated counterpart. Any observed differences in metabolism in the germ-free animal may thus be secondary to alterations in physiological processes and do not necessarily indicate the direct action of specific micro-organisms.

*Characteristics of germ-free chickens*

As might be expected, the germ-free bird is characterised by underdevelopment of the humoral and cellular defence mechanisms. In 11-week-old germ-free chickens, Wostmann and Gordon (1958) found a greatly decreased concentration of serum  $\gamma$ -globulin and some decrease also in the  $\beta$ -globulin fraction. At 5 weeks of age the ileocaecal lymph nodes are very underdeveloped (Gordon, Wagner and Wostmann, 1958) and the scattered reticulo-endothelial cells in the ileal mucosa are drastically reduced in number (Gordon and Bruckner-Kardoss, 1958-59). The bursa and thymus are only slightly smaller in germ-free birds (T. G. Taylor, unpublished).

It is questionable whether these morphological changes are likely to affect the nutritional processes. Of more concern to the nutritionist are the alterations in intestinal structure that have been observed in

TABLE 1

*Mean weight and length of small intestine in groups of germ-free and conventional chicks at 4 weeks of age*

Type of diet	No. of chicks	Type of chick	Body wt. (g.)	Intestinal wt. (g./g. body wt.)	Intestinal length (cm./g. body wt.)
Natural	17	Germ-free	265	0.033	0.32
	17	Conventional	255	0.043	0.35
Purified	27	Germ-free	327	0.027	0.24
	27	Conventional	350	0.032	0.25

germ-free chickens. As the results of our unpublished experiments given in Table 1 show, the small intestine of the germ-free bird is considerably reduced in weight; the length is less noticeably reduced, hence the lower weight must be accounted for by a thinning of the intestinal wall. Histological studies (Gordon and Bruckner-Kardoss, 1961) have shown little difference in the muscular elements of the intestine but a marked reduction of the lamina propria in germ-free chickens, particularly noticeable in the "core" of the villi, which leads to an overall reduction in villus size. Thus although the proportion of mucosal cells relative to other tissue is higher in the germ-free intestine, the total absorptive surface area is less because the dimensions of the villi are smaller. Such structural differences might be expected to give rise to differences in absorption of nutrients from the small intestine, but there is little information yet available on this point. Studies with rats and mice indicate that passive absorption of, for instance, xylose is significantly increased in germ-free animals, whereas active transport of substances such as  $\text{Na}^+$  and glucose is not altered (Heneghan, 1963). No such evidence exists for the chicken, and it remains a matter for speculation whether or not the presence of an intestinal flora affects the passage of essential nutrients across the gut wall in the bird.

In germ-free rodents a gross enlargement of the caecum frequently



occurs, and passage of fluid from the tissues into the caecum leads to considerable haemodynamic disturbance. This phenomenon has never been observed in birds. The caeca of germ-free chickens are slightly smaller than those of conventional birds but show no obvious abnormality. Other major physiological changes that have been observed in germ-free mammals are a lowered cardiac output and reduced arterial blood flow to the liver. There is no corresponding information about the chicken.

### *Nutritional and Metabolic Differences between Germ-free and Conventional Chickens*

Nutritional studies with germ-free chickens have so far been comparatively few. Available information regarding the influence of the gut flora on avian digestion and metabolism is summarised here although, in view of the complications already discussed, the precise mechanism of the observed effects is not always clear.

### *Digestive Enzymes*

In the course of a study of pancreatic enzymes, Lepkovsky, Wagner, Furuta, Ozone and Koike (1964) measured proteases, amylase and lipase in the contents of the alimentary tract of 10-week-old germ-free and conventional chickens. Little difference was noted in the amounts of the enzymes present in the contents of the upper and lower intestine. There was some evidence of lower concentrations of proteases and amylase, and higher amounts of lipase in the caecal contents of conventional birds. Protein nitrogen was almost completely absent from the germ-free caecal contents, a finding compatible with the increased level of proteases as well as with the absence of bacterial protein. These results give no indication that proteolytic, amylolytic or lipolytic activity in the small intestine is affected by the presence or absence of a microflora; whether or not the alterations in the products of caecal digestion are of major importance in the nutrition of the bird is open to question.

### *Vitamin Economy*

In many species of animal there is evidence that vitamins of the B complex are synthesised by bacterial action in the alimentary tract. So far only vitamin B<sub>12</sub> has been studied in the germ-free chick. On a purified diet containing cyanocobalamin the caecal content of vitamin B<sub>12</sub> was a hundred times greater in conventional than in germ-free chicks. Large quantities of vitamin B<sub>12</sub>-like factors were present in the conventional caecal contents, but none was detected in those from germ-free birds (Coates, Gregory, Porter and Williams, 1963). These results give clear evidence for the microbial synthesis of vitamin B<sub>12</sub> and its

TABLE 2

*Mean liver reserves of vitamin A in groups of germ-free and conventional chicks at 4 weeks of age*

Type of diet	No. of chicks	Type of chick	Vitamin A ( $\mu\text{g./g. liver}$ )
Natural	4	Germ-free	51
	12	Conventional	32
Purified	12	Germ-free	105
	24	Conventional	62

analogues in the alimentary tract. Responsible organisms have not yet been investigated, nor is it known whether any of the synthesised vitamin B<sub>12</sub> is available to the bird without ingestion of its droppings.

### *Volatile Fatty Acids*

In the ruminant the volatile fatty acids that arise from microbial fermentation in the rumen form an important source of energy for the host animal. In the chicken Hill, Annison and Noakes (1965) detected high concentrations of volatile fatty acids in caecal contents. Acetic and propionic acids formed the major components, although several other short-chain fatty acids were also present. All the individual acids were detected in portal blood, but peripheral blood contained mainly acetic acid and a little formic acid. In comparisons of germ-free and conventional chickens the volatile fatty acid concentrations in m mole/kg. caecal contents were, respectively, 0.30 and 10.0 at 2 weeks of age and 3.5 and 70.0 at 5 weeks of age. In spite of these enormous differences in caecal content, the blood levels of volatile fatty acids (mostly acetate) were much the same in germ-free and conventional birds. It can be concluded, therefore, that the short-chain fatty acids of chicken blood are not of microbial origin.

### *Cholesterol Metabolism*

There is evidence from studies *in vivo* and *in vitro* that gut micro-organisms can effect the catabolism of cholesterol and bile acids. In germ-free animals elevated plasma cholesterol levels are frequently observed, possibly because in the absence of a microflora the cholesterol and bile acids eliminated in the bile are not degraded but remain

available for re-absorption lower down the intestine. Results of our own experiments with chicks given a purified diet (without added cholesterol) are given in Table 3 (Coates, Harrison and Moore, 1964). On every occasion the germ-free birds had higher levels of cholesterol in the plasma, although the increase over the controls did not always reach statistical significance. The rise in plasma cholesterol was, however, always accompanied by a corresponding fall in cholesterol content of the liver, so that the net result of the absence of a microflora was a shift in the partition of cholesterol between blood and liver. An explanation for this finding is not at once obvious and may lie in the physiological differences between germ-free and conventional

TABLE 3

*Cholesterol content of the liver and plasma of 4-week-old germ-free and conventional chicks given a purified diet containing starch*

Expt No.	Type of chick	No. of chicks	Body wt.(g.)	Cholesterol content (mg.)			
				Per 100 ml. plasma	Total in plasma*	Whole liver	Liver+ plasma
1	Germ-free	24	345	155	32.1	25.6	57.7
	Conventional	24	327	96	18.8	36.8	55.6
2	Germ-free	24	370	181	40.2	26.1	66.3
	Conventional	24	346	171	35.5	32.3	67.8
3	Germ-free	16	342	151	30.8	20.3	50.8
	Conventional	24	332	119	23.8	33.0	56.8
4	Germ-free	22	333	171	34.2	21.7	55.9
	Conventional	22	319	163	31.3	22.4	53.7

\* Assumed that total plasma equals 6 per cent of body weight.

chickens. For instance, differences in rate of blood flow to the liver or other organs might affect the amount of cholesterol deposited there; an altered lipid or protein metabolism in the germ-free bird might influence the cholesterol content of plasma, where it occurs in the form of a lipoprotein. Alternatively if cholesterol is less efficiently eliminated in the absence of a gut flora, the consequently higher plasma content might suppress *de novo* synthesis of cholesterol in the liver.

In the foregoing experiments starch was the main source of carbohydrate in the diet. Replacement of starch by sucrose led to elevated plasma cholesterol levels in both the germ-free and conventional groups (Table 4). Liver content of cholesterol was also very high in the germ-free birds given sucrose. It appears from these results that a high content of sucrose in the diet leads to high levels of cholesterol in the blood and liver of chickens, but this effect is to some extent counteracted by the presence of a gut microflora.

#### *Growth-promoting Effect of Antibiotics*

It is apparent from the figures in Table 4 that growth of the conventional birds given sucrose was very poor. Since the germ-free birds

grew normally on the sucrose diet, it must be assumed that some component of the gut flora was responsible for the growth depression in a conventional environment. A similar finding has been reported by Eysson and de Somer (1963) who observed enteritis, malabsorption of nutrients and a thickening of the gut wall to accompany the growth depression, which could be prevented by oral antibiotics. These workers were unable to reproduce the whole syndrome in germ-free chicks by contamination with a pure or mixed flora of thirty-five strains of intestinal bacteria; the effects were partially induced, however, by contamination with a gram-positive coccus (Eysson and de Somer,

TABLE 4

*Effect of different dietary carbohydrates on liver and plasma cholesterol content of groups of sixteen germ-free and conventional chicks*

Type of diet	Type of chick	Body wt. (g.)	Cholesterol content (mg.)			
			Per 100 ml. plasma	Total in plasma*	Whole liver	Liver + plasma
Starch	Germ-free	313	128	24.0	23.6	47.6
	Conventional	350	116	24.4	28.9	53.3
Sucrose	Germ-free	359	143	30.8	30.3	61.1
	Conventional	273	122	20.0	25.3	45.3

\* Assumed that total plasma equals 6 per cent of body weight.

1964). Similar findings have been reported by Huhtanen and Pensack (1964), who later isolated a strain of *Streptococcus faecalis* that caused some growth depression in germ-free chicks not given dietary antibiotics (Pensack, private communication).

### *Bacteriological Aspects*

The failure of both these groups of workers to reproduce the syndrome with full severity by monocontaminating germ-free birds with what appeared to be the causative organism raises the question of the balance of the intestinal flora and the relationship of individual micro-organisms to each other. The use of germ-free chicks as "test beds" for determining the growth stimulating or depressing effects of gut micro-organisms has been of undoubted value in some instances but, in others, care must be taken in interpreting results. By comparing the growth of germ-free chicks fed on a diet deficient in some nutrient, say a vitamin, with that of conventional chicks fed on the same diet, it is possible to show whether the gut flora provides the missing nutrient in sufficient concentration, in a suitable form and in a site in the gut from which it can be absorbed. Similar tests can show whether micro-organisms are responsible for growth depression. However, difficulties arise when it is required to know which constituent of the gut flora is implicated. In the past, as indicated in previous paragraphs, pure cultures of bacteria have been established in germ-free chicks and the

effects noted. In these circumstances the organisms develop in the absence of the extremely complex flora present under "normal" conditions and are not exposed to the inhibitory and/or stimulatory interactions which occur in mixed populations. Furthermore, such a technique may prove unsatisfactory for certain bacteria which will not establish themselves in the gut unless other bacteria are present (e.g. certain strict anaerobes may depend on the low oxidation-reduction conditions created by less sensitive types). The point may be illustrated by reference to *Clostridium welchii* and its implication in the growth depression of young chicks. During past years evidence has been put forward that the type of depression relieved by dietary penicillin may be due to this organism. To provide additional evidence, the organism has been introduced into germ-free chicks and the observed depression interpreted as further confirmation (Lev and Forbes, 1959). Under these conditions *Cl. welchii* invades the whole of the intestine and caeca and persists in high numbers for at least 4 weeks. However, in conventional chicks fed on the same diet the organism is reported to be present only in the caeca in high numbers and virtually to disappear after only a few days. It seems likely that the pattern of colonisation of the gut by *Cl. welchii* is markedly influenced by other constituents of the "normal" gut flora: it is tempting to enquire why an organism which is believed to cause a certain degree of depression in the growth of "normal" chicks as a result of temporary colonisation of the caeca, only causes the same degree of depression when introduced into germ-free birds in which colonisation is both more extensive and permanent.

One means of overcoming these difficulties is being examined at present. This involves the testing of possible adverse or beneficial effects of single cultures of gut micro-organisms by introducing them into birds simultaneously with a complex "background" flora previously shown not to exert the particular effect being studied. There are considerable practical difficulties involved in such techniques, however, and these are aggravated by the relative lack of information on what constitutes the "normal" gut flora of chickens.

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## PART III

### CALCIUM METABOLISM AND EGGSHELL FORMATION

## THE FUNCTIONAL ANATOMY OF THE AVIAN SHELL GLAND

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### *Synopsis*

THE ANATOMY of the blood supply to the shell gland of the domestic hen has been investigated using injection methods based upon rubber latex and indian ink injection masses. The ramifications of the blood vessels on the shell gland surface and the relationships of the vascular branches to the layers of the gland wall and the glandular folds have been described. Examination of the vascular casts and of sections of injected shell glands indicated that there was considerable fluctuation in vascular volume between the active and the quiescent states of the gland. This knowledge of the vascular anatomy was used to devise a technique for sampling shell gland venous blood. Using this technique some preliminary experiments have been performed, measuring the pH of shell gland venous, systemic venous and systemic arterial blood during the greater part of the shell secretion cycle.

### *Introduction*

The main function of the shell gland, the production of a calcified shell on the surface of the shell membranes, is carried out by the secretory epithelium of the gland. This consists of two parts: firstly, the superficial ciliated epithelium bordering the lumen of the oviduct and secondly, the layer of tubular glands underlying this epithelium. These two layers, which together constitute the mucous membrane, are formed into many flat, leaf-shaped glandular folds, so greatly increasing the area of secretory epithelium. Beneath the mucous membrane the remainder of the wall of the shell gland is made up of five consecutive layers: a submucosal connective tissue layer; a circular muscle layer; an intermuscular connective tissue layer; a longitudinal muscle layer; and an external layer of peritoneum.

Ramifying through all these layers of the gland is a complex system of blood vessels whose role is to supply the secretory epithelium not only with the oxygen and nutrients necessary for the functioning of the gland but also with the materials which are to be secreted to form the



shell. As there is no storage of these secretory products in the gland during the resting phase, they must be made available at the time of shell secretion thus necessitating an adequate and efficient blood supply. The first part of the present account consists of a description of the anatomy of the blood supply of the shell gland. This examination of the vasculature was carried out as a preliminary to the investigation of certain aspects of the physiology of shell secretion.

### *Materials and Methods*

The methods which were used to investigate the vascular anatomy consisted in the first place of the injection of coloured latex followed by acid maceration of the casts; and in the second place of injection of indian ink followed by clearing or sectioning of pieces of the injected glands. In all cases the blood was flushed out of the bird by a preliminary injection of warm physiological saline and all injections were carried out at pressures as near as possible to the normal mean arterial pressure in order that the casts should be truly representative of the blood vascular system. Arterial injections were performed through the aorta and venous injections through the inferior vena cava (Hodges, 1965). The birds used in the injection experiments were mature Light Sussex hens.

### *Results*

#### *Anatomical Studies*

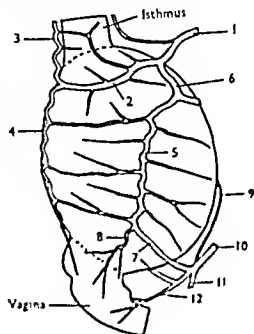
The main blood vessels supplying the glandular wall occur in the layer of consecutive tissue between the two muscle layers and are thus plainly visible externally. These major vessels are supplied from three points (Fig. 1).

Firstly, there is the hypogastric artery (1), a branch of the left sciatic artery, which reaches the gland at a point at the front of the

FIG. 1

Lateral side of the hen's shell gland showing the arteries supplying the gland and the normal pattern of arteries running in the intermuscular connective tissue layer.

1. Hypogastric artery.
2. Anterior uterine artery.
3. Inferior oviducal artery.
4. Inferior uterine artery.
5. Lateral uterine artery.
6. Superior uterine artery.
7. Posterior uterine arteries.
8. Utero-vaginal artery.
9. Middle uterine artery.
10. Left internal iliac artery.
11. Left palmar artery.
12. Left pelvic artery.



dorsal surface. Secondly, there is a pair of posterior uterine arteries (7), branches of the left internal iliac artery, and these impinge upon either side of the caudal end of the gland. Thirdly, there is a smaller artery connecting the anterior uterine artery (2) with the inferior oviducal artery (3) running through the ventral mesentery of the oviduct. These arteries divide up on the surface of the gland to form the major vessels supplying the gland, the anterior uterine artery (2), the superior (6) and inferior uterine arteries (4), the lateral uterine artery (5) and

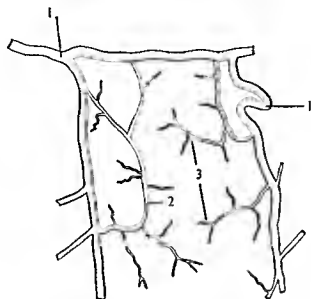


FIG. 2. Small section of the lateral face of a shell gland showing some of the main arteries:

1, together with their primary branches; 2, and the beginning of the secondary branches; 3, most of which disappear by passing through the circular muscle layer. All these vessels lie in the inter-muscular connective tissue layer. Approx.  $\times 2$

many smaller branches (Freedman and Sturkie, 1963; Hodges, 1965). These major arteries and their primary branches are interconnected in a network over the whole surface of the gland.

In nearly every instance there is a vein accompanying each artery, whether large or small (Hodges, 1965).

As may be seen in Fig. 2, the main arteries (1) lying between the muscle layers divide frequently to give primary branches (2). These divide again to give secondary arterial branches (3), the majority of which pass through the circular muscle coat into the submucosa and are lost to view. In the submucosa these secondary branches form a network which gives off tertiary, arteriolar branches supplying the leaf-shaped folds of the secretory epithelium. The tertiary branches pass up through the connective tissue corium of the glandular folds and, break up into capillaries which pass outwards between the tubular glands, giving rise to an anastomosing capillary network which lies just beneath the ciliated epithelial layer. The venous return almost exactly parallels the arterial system.

The overall pattern of the blood supply and its relationship to the

different parts of the shell gland wall are illustrated diagrammatically in Fig. 3, while Plate 16A portrays the actual relationships of the blood vessels as they are revealed in cleared sections of portions of ink injected glands.

Examination of the anatomical specimens has given rise to some interesting possibilities. The majority of the glands injected with latex were in the active condition, i.e. they contained an egg, whilst the

1. Peritoneum.
2. Longitudinal muscle layer.
3. Intermuscular connective tissue layer.
4. Main blood vessels.
5. Primary blood vessel branches.
6. Circular muscle layer.
7. Network of secondary blood vessel branches.
8. Submucosa.
9. Tertiary branches passing up through the corium of the glandular fold.
10. Layer of tubular glands through which pass capillary branches.
11. Internal ciliated epithelium.
12. Capillary network underlying ciliated epithelium.

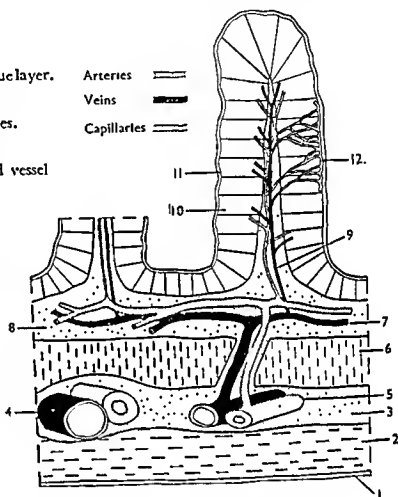
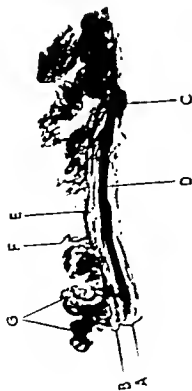


FIG. 3. Diagram showing the different parts of the shell gland blood supply and their relationships to the layers of the gland wall.

injection was occurring. But a few of them were in the quiescent state. A comparison of the weights of some of the latex casts of these two types of glands showed that there was a considerable difference in weight between the two categories. Thus the mean values were: active glands (14 glands)  $1.85 \text{ g.} \pm 0.34 \text{ S.D.}$ ; quiescent glands (7 glands)  $1.08 \text{ g.} \pm 0.21 \text{ S.D.}$  When subjected to the "t" test these figures were significant at the 5 per cent level. Thus it can be assumed that there is a correlation between the weight of the latex cast and the vascular volume of the shell gland, and there is on average an increase of 42 per cent between the vascular volume of an active gland over a quiescent gland.

It was noted in the latex casts of the quiescent glands that the capillary beds of the epithelial folds were hardly ever filled by the latex



Thick section of portion of cleared, ink injected shell gland demonstrating the relationships of the vascular system to the different parts of the gland wall

*B.* Muscular layer and intermuscular connective tissue layer. *B.* Submucosal layer. *C.* Main blood vessel. *D.* Primary branch blood vessel running in the muscular layers. *E.* Secondary branch vessels in the submucosa. *F.* Tertiary branch vessel supplying glandular fold. *G.* Glandular folds.



Single glandular fold of the shell gland

*A and B.* Tertiary  
*A.* Capillary network

and this observation was confirmed in the ink-injected glands, the ink rarely penetrating further than the tertiary arterial branches entering the glandular folds. Sections taken from the latter glands showed that the epithelial capillaries still contained erythrocytes, indicating that the preliminary saline injection had failed to enter these vessels. Examination of sections of ink injected active glands showed that these capillary beds, although often incompletely filled by the ink, had been entirely washed free of blood cells by the saline.

It appears from these results that in the quiescent state the blood flow through the secretory epithelium is considerably reduced, probably by contraction of the arterioles supplying the glandular folds. Although there is little or no flow through the capillary beds during injections of quiescent glands, the fact that the ink flows easily through the gland indicates the possible presence of arterio-venous anastomoses at the level of the secondary blood vessel branches in the submucosa. However, no such anastomoses have yet been found.

### *Physiological Studies*

Using the information obtained from the anatomical study it has been found possible to cannulate a shell gland vein and thus obtain samples of shell gland blood over periods of several hours. The posterior end of the inferior oviducal vein was cannulated (Hodges, 1965). The results reported here are those from some preliminary experiments performed upon nine birds (Sterling White Links). The birds were anaesthetised and the shell gland vein and the left sciatic artery and vein were cannulated. Small samples of blood were taken at hourly or half-hourly intervals throughout the egg-laying cycle and the pH of the samples was measured with a Radiometer micro-pH electrode. No bird was sampled throughout the whole egg-laying cycle; the longest single period was one of  $11\frac{1}{2}$  hr.

The fluctuations in pH which occur before and after an egg has entered the shell gland can be seen in Fig. 4. These results were taken from a single experiment. The difference between the arterial blood pH and that of the shell gland vein on average doubled in value after the egg had entered the shell gland. Both before and after shell gland activity had begun the pH of the venous blood of the shell gland was much closer to that of arterial blood than to that of systemic venous blood. This was possibly due to a high rate of blood flow through the shell gland. The marked fluctuations in the systemic venous blood were apparently caused by stagnation effects in the leg owing to the sciatic artery being cannulated. These effects were partially overcome in later experiments by inserting the venous cannula in the direction of blood flow rather than against it. The overall rise in pH which

An overall picture of the fluctuations in the pH difference between arterial and shell gland venous blood can be seen in Fig. 5. This is a composite picture of all the experiments. The difference during the

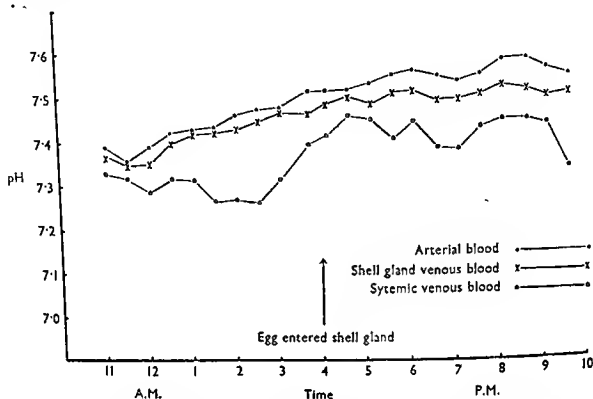


FIG. 4. The pH values of hen's blood in relation to the early stages of shell formation. Based upon one experiment only.

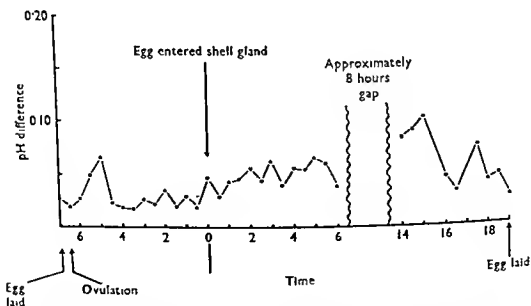


FIG. 5. The pH difference between systemic arterial and shell gland venous blood during shell formation; preliminary experiments. Composite picture obtained from nine separate experiments.

quiescent period, apart from one sharp peak about  $1\frac{1}{2}$  hr. after ovulation, was fairly small. Once the egg had entered the shell gland this difference began to increase steadily. Owing to the fact that no bird was successfully cannulated for the full cycle no results were obtained for the 8 hr. in the middle of the shell secretion period. This is the time of most

active shell secretion. Towards the time of laying the pH difference fell sharply to the resting level from a figure more than three times as large as this resting level.

### *Discussion*

The investigation into the vascular supply of the shell gland was undertaken mainly to provide information concerning the pattern of distribution of arteries and veins on the surface of the gland, thus enabling these blood vessels to be readily cannulated when studying the physiology of shell secretion. When carrying out such cannulations several factors have to be borne in mind. Firstly, care must be taken to cannulate a vessel which only contains pure shell gland blood. This factor can be considered as a possible criticism of the work of Hunsaker and Sturkie (1961) who cannulated veins on the postero-lateral surface of the shell gland in order to obtain blood for calcium estimations. Personal experience of their operative technique has shown that the incision frequently exposes the utero-vaginal vein (Hodges, 1965) which drains not only uterine tissue but also part of the vagina. However, Hunsaker (1959) has stated that vessels leading from the vagina were clamped off in an attempt to reduce contamination of the shell gland blood. Secondly, the shell gland is notoriously susceptible to surgical interference. Sykes (1953) has shown that the presence of a loop of thread in the wall of the shell gland causes eggs to be laid with little or no shell. Thus cannulae tied into the wall might upset the shell secretion mechanism in a similar manner. Thirdly, the slightest surgical interference with the highly vascular wall of the gland, such as the insertion of a needle and thread, causes an effusion of blood in the tissues which obscures the site of operation. Generally speaking, it is only necessary to cannulate shell gland veins for this type of physiological investigation as the main blood supply to the gland is through the hypogastric branch of the sciatic artery, and cannulation of the sciatic artery will give arterial blood of similar composition to that entering the shell gland. The vein which has been used in these experiments not only appears to overcome all the above-mentioned objections but is also comparatively easy to cannulate.

The major constituent of a shell is calcium carbonate and this is presumably secreted by the glandular epithelium in the form of calcium and bicarbonate ions. Beadle, Conrad and Scott (1938) have shown that the shell gland secretion contains calcium and large amounts of bicarbonates. When investigating the variations in shell gland blood composition during shell secretion the most obvious measurements to take are blood calcium and blood bicarbonate or carbon dioxide concentrations. For these preliminary experiments the measurement selected was that of blood pH as it is simply and rapidly measured and is correlated with the blood bicarbonate and carbon dioxide concentrations. Thus if the shell gland is extracting bicarbonate ions from its

blood supply then the  $pH$  of the venous blood of the shell gland will fluctuate in contrast to that of arterial and whole body venous blood, according to the rate of secretion.

Although the fluctuations in blood  $pH$  which have been demonstrated almost certainly reflect the process of shell secretion, their interpretation is made difficult by the number of factors likely to have an effect on  $pH$  which are involved in shell secretion. Firstly, there is the secretion of bicarbonate for shell formation. Secondly, the increased metabolic activity of the secretory epithelium during secretion will cause an increased carbon dioxide production, again affecting the  $pH$ . Thirdly, there is the complicating factor of the fluctuations in blood flow which apparently take place during the cycle of egg formation.

In order to gain an understanding of how these fluctuations are related to shell secretion, the  $pH$  must be compared with the bicarbonate and carbon dioxide concentrations of the blood and also with the stage of secretion.

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# THE ENDOCRINE CONTROL OF CALCIUM METABOLISM IN THE FOWL

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## *Synopsis*

THE INTERRELATIONS between the various hormones concerned in regulating calcium metabolism in the laying hen are reviewed. The oestrogen-induced hypercalcaemia of laying birds is related to the transport of yolk proteins rather than to the provision of calcium for shell formation. Oestrogens, acting in synergism with androgens, are also concerned in the induction of medullary bone, which acts as a labile reserve of calcium for the provision of calcium for egg shell formation when the rate of absorption from the gut is inadequate. The mechanism by which destruction of medullary bone is brought about is discussed, and it is suggested that the parathyroid hormone is responsible. The cessation of egg production in hens fed a calcium-deficient diet is thought to be due to a reduction in gonadotrophin secretion by the anterior pituitary, and this mechanism serves to protect the skeleton from excessive depletion. It is suggested that the inhibition of pituitary gonadotrophin secretion is mediated via the hypothalamus, and that the latter is sensitive to a reduction in the level of ionic calcium in the blood. It appears that birds which continue to secrete gonadotrophins in spite of a serious fall in the level of their blood calcium, may be particularly prone to the condition of "layers' cramp".

## *Introduction*

Calcium metabolism in the laying hen proceeds at a very high rate, mainly in relation to the formation of the egg shell, which normally contains between 1.5 and 2.5 g. calcium, the actual amount depending on the size of the egg and the thickness of the shell. Control of this intense metabolism is regulated by a number of different endocrine glands, including the ovary, the parathyroid, the anterior pituitary and possibly others, and the interrelations between the hormones produced by these glands provide the subject matter for this paper.

*The role of sex hormones*

In the past the greatest emphasis has been placed on oestrogens in relation to calcium metabolism in laying birds and the effect of these hormones on the level of plasma calcium is certainly most striking. It is now recognised, however, that the oestrogen-induced hypercalcaemia observed in laying birds is related, not to the provision of calcium for shell formation, but to the transport of yolk proteins, since it occurs also in female frogs, fish and snakes during the breeding season, none of which lay eggs with calcified shells (Urist, 1959).

Nevertheless, oestrogens in conjunction with androgens play an important role in bone metabolism in laying hens, and it is this role that will be stressed. Under the combined influence of these sex hormones a whole new system of secondary bone develops in the marrow cavities of many of the bones of the pullet during the 10-14 days before the first egg is laid and it persists throughout the laying season. This medullary bone, as it is called, acts as a readily available reserve of calcium which is mobilised for egg shell formation whenever the level of absorption from the gut is insufficient. Another effect of oestrogen, again acting in synergism with androgen, is that it greatly enhances the absorption of both calcium and phosphorus from the intestinal tract. Presumably these minerals are used for the calcification of the medullary bone. (Neither oestrogen nor androgen alone has an appreciable effect on calcium and phosphorus absorption.)

*The cyclic changes in medullary bone*

The medullary bone undergoes cyclic changes during the formation of the egg, phases of intense bone formation alternating with phases of equally intense bone destruction. These phases are most marked in the pigeon but they occur also in the domestic fowl. The bone-forming stage is initiated following oviposition and it continues at a high rate during the first few hours of calcification of the next egg of the sequence. As shell formation advances the rate of bone formation declines and a phase of bone destruction begins which continues right up to the time when the egg is laid.

*The role of the parathyroid*

It is interesting to speculate as to the mechanism by which the rapid reversal of the bone-forming and bone-destroying phase during the egg cycle is brought about. Riddle, Rauch and Smith (1945) and Urist (1959) have suggested that variations in the level of plasma oestrogen during the egg cycle are responsible for the changes in the medullary bone, since it requires a constant oestrogenic stimulus for its maintenance. It seems much more plausible, however, that changes in the medullary bone are due to changes in parathyroid activity. This theory is supported by the rapidity with which the changes occur and

by the similarity between the histological picture observed in medullary bone at the height of shell calcification and in bone under the influence of parathyroid hormone. Furthermore it has been shown that there is a fall in the level of diffusible calcium in the blood during shell formation, so that the stimulus for parathyroid activity is present (Taylor and Hertelendy, 1961).

Nothing is known of the possible involvement in egg shell formation of calcitonin, the recently discovered hypocalcaemic factor of the parathyroids (Copp, Cameron, Cheney, Davidson and Henze, 1962; Copp, 1964) or of thyrocalcitonin, a hormone with similar properties originating in the thyroid (Hirsch, Gauthier and Munson, 1963; Foster, Baghdiantz, Kumar, Slack, Soliman and MacIntyre, 1964), but if they occur in birds one would not expect them to do more than prevent the plasma calcium "overshooting" following oviposition.

#### *The role of the pituitary and the hypothalamus*

Taylor, Morris and Hertelendy (1962) have recently demonstrated an interesting relationship between calcium metabolism and the anterior pituitary gland in the laying hen. Birds of a strain which, when placed on a low-calcium diet, normally cease production in 10-14 days after laying 5-7 eggs, were shown to continue laying when injected daily with an extract of crude avian pituitary material starting on the fifth day after the calcium-deficient diet was introduced. The 3 injected birds laid, respectively, 5, 5 and 6 eggs in the 5 days following the start of the injections while the control (uninjected) birds laid 1, 3 and 1 eggs, respectively, during the same period. The most reasonable explanation for these observations seems to be that the amount of gonadotrophin released from the anterior pituitary is reduced in an acute state of calcium deficiency and it was suggested that this "pituitary cut-off" mechanism serves to protect the skeleton from excessive depletion.

The mechanism by which secretion of pituitary gonadotrophins is inhibited under these conditions is not known but an hypothesis now being considered is that the effect is mediated via the hypothalamus. In the experiments of Taylor *et al.* (1962), the mean level of diffusible calcium in the plasma of the injected hens at killing was at least 2 mg./100 ml. lower than in untreated controls fed a high-calcium diet and there can be little doubt that the plasma level would have been even lower during active shell calcification. The hypothalamus is sensitive to many chemical and environmental stimuli and it may well be influenced by the level of ionic calcium in the plasma.

If there is a critical level of ionic calcium in the plasma below which secretion of a "gonadotrophin releasing factor" by the hypothalamus is inhibited it is possible that this level is reached in some birds during shell calcification even when a high-calcium diet is fed. The effect of this would be to reduce gonadotrophin secretion, which would in turn

reduce the rate of follicle growth and thus reduce the rate of ovulation. This postulated "pituitary cut-off mechanism" might therefore be a factor in determining the level of egg production of individual birds. A reduced rate of secretion of gonadotrophin during shell calcification would also be expected to reduce the rate of oestrogen secretion, one effect of which might be to stimulate the resorption of medullary bone as envisaged by Riddle *et al.* (1945) and Urist (1959).

The sensitivity of the "pituitary cut-off" mechanism is somewhat variable, and in high-producing strains it appears to be relatively insensitive at the onset of lay when gonadotrophin secretion is probably maximal. Taylor and his collaborators (1962), have suggested that, in selecting for a high rate of egg production, breeders may unconsciously produce a bird in which the "cut-off" mechanism is of low sensitivity, one effect of which is that the birds are more prone to the condition of "layer's cramp".

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## SHELL STRENGTH

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THE VARIOUS basic methods of measuring shell strength, namely by impact, crushing, snapping and deformation, are mentioned. The errors of the methods and the relationship between methods are briefly discussed.

With batches of mixed eggs a significant correlation is usually found between strength and thickness, but if eggs from individual birds are considered, the correlation may vary from highly significant to non-significant. Furthermore, thickness at different points of the same shell can vary greatly from mean thickness.

Translucent areas of a shell are weaker than opaque areas on the same shell as measured by the needle method. There is also some evidence that when the snapping method is used, the membrane affects the strength.

Studies of shell damage show that shells sometimes crack cleanly and sometimes crumble. The cracks run through crystals as well as between them and twinning planes may appear in the calcite crystals before any visible damage is seen.

*Introduction*

Shell strength has been measured for years but it is only recently that really determined efforts have been made to study fundamental problems relating to it. This paper will be concerned with a few of our major findings at Reading.

*Methods*

Methods of measuring shell strength may be divided into four main groups (Tyler, 1961). First there is the impact or cracking method which is based on the falling ball technique. The ball may be allowed to fall from ever increasing heights, it may be allowed to fall from a constant height or balls of ever increasing weight may be dropped. Within each of these methods the balls may be dropped on one spot or on different spots. In our experience the most promising method

seems to be the one in which the ball is dropped from a fixed height on to a series of points close together but in a line about 1.5 cm. long. This does at least ensure that most of the cracks produced run approximately along this predetermined line instead of running in any direction (Tyler and Geake, 1963, 1964a).

The second group consists of the crushing methods and these depend upon the concept of a steadily increasing load applied to the egg. It has recently been shown that a flat plate, a flat-ended peg, a round-ended peg or a needle give relatively the same result and despite the fact that the needle pierces the shell it can be regarded as the extreme limit of the crushing technique (Tyler and Geake, 1963, 1964c).

Another method is that in which a strip of shell is firmly held and then subjected to an increasing load until it snaps. Obviously, this method has to be standardised so that pieces of shell of equal widths are used and the point of application of the load must be a fixed distance from the point where the shell is held (Almquist and Burmester, 1934). Work has started on this method but apart from a few points mentioned later the results have not yet been published.

More recently an apparatus which deforms the egg without breaking it has been designed (Schoorl and Boersma, 1962). This is basically the same idea as that used by Brooks and Hale (1955), except that the new apparatus merely measures deformation under one standard load of 500 g. The great advantage of this method is that the egg is not broken, but it is difficult to judge exactly what is being measured. Is it general strength or strength at the broadest part to which the pressure is applied? Clearly, these points will need to be studied in greater detail.

Whatever method is used it is obviously advantageous if more than one comparable reading can be taken on the same egg. Firstly, because replicate readings are statistically better than single ones in that a mean is more reliable than a single reading but also because replication makes possible the calculation of the error of the method. By making use of the fact that egg shells are relatively uniform in thickness and composition around any given latitude, it is possible to make replicate measurements on fairly uniform areas of shell. Using impact methods, two measurements may be made at the waist, while with the needle and snapping methods up to eight measurements are possible. Theoretically an infinite number is possible with the deformation apparatus (Tyler and Geake, 1964a, b, c).

The errors of the various methods have been calculated and it seems that impact methods have a far greater error than the crushing methods (Tyler and Geake, 1964a, b). The error of the crushing method is about equal to or perhaps slightly greater than the snapping method. The deformation method gives the lowest error of all but this is misleading because in effect the deformation refers to the deformation around a complete latitude and hence squeezing the egg between any

two opposite points on this latitude and then between two other points on the same latitude is not the same kind of duplicate reading as when the needle is used to pierce two entirely separate holes on one latitude or when two entirely different pieces of shell are snapped (Table 1).

TABLE 1

*Measurement of egg shell strength: comparison of different methods all applied to the same 20 eggs*

Method	Error	Spread
Piercing	1.53	28
Snapping	1.19	18
Deformation	0.66	27
Analysis of variance		Variance
Eggs		$V_E$
Treatment		$V_T$
Error		$V_e$
<hr/>		
Total		

$$\text{Error} = \sqrt{\frac{V_e}{40}} \times \frac{100}{M}$$

$$\text{Spread} = \sqrt{V_E} \times \frac{100}{M}$$

The data collected can also be used to measure the spread (coefficient of variation) of results over a series of eggs and here the snapping method gives a much lower value than the other methods. This is a disadvantage when looking for differences between eggs (Table 1).

It should also be noted, that the deformation method is not very suitable for fundamental studies but that it may well prove to be the most suitable method for testing eggs in large numbers for breeding purposes and for practical information in relation to the transport of eggs.

#### *Relationship between methods*

Mention has already been made of the relationship between the different crushing methods, and this is not surprising if they are truly similar methods. It can now be reported that there is very good agreement between deformation and snapping, deformation and piercing and deformation and line cracking. There is also good agreement between snapping and piercing. There is, however, no agreement between deformation and spot cracking. This latter result is due to the fact that spot cracking is probably the least precise and reproducible of methods (Table 2).

#### *Relationship of strength to thickness*

Most early workers showed that strength and thickness could be correlated but that the correlation coefficients were of such a magnitude

that obviously other factors in addition to thickness were involved (Tyler, 1961).

Recently a precision instrument has been built for use with needle and snapping methods and the deformation apparatus has become available. Results with these new pieces of apparatus show a closer relationship between thickness and strength and in one experiment the correlation coefficient for deformation against thickness was as high as 0.95 for 20 eggs. It has been shown, however, that the correlation between strength and thickness can vary a great deal amongst individual birds: some show a very high correlation others a very low one (Tyler and Geake, 1964d).

Another aspect of this matter arises from the fact that egg shell thickness is not uniform over the whole surface of the egg. Variations around any latitude are very small, but variations from pole to pole

TABLE 2

*Measurements of egg shell strength: relationship between different methods using 20 eggs for each comparison*

Methods	Correlation coefficient
Deformation v. Piercing	0.72***
Deformation v. Snapping	0.70***
Snapping v. Piercing	0.60**
Deformation v. Cracking (line)	0.66**
Deformation v. Cracking (spot)	0.14 NS

Deformation values were expressed as their reciprocals.

can be very large. Individual birds may each show a different pattern of shell thickness and while some birds adhere to the same pattern over long periods, others change their patterns (Tyler and Geake, 1965). Therefore, shell thickness measurements around a particular latitude do not necessarily give an indication of overall shell strength and measurements of mean shell thickness similarly fail to indicate shell strength at a particular point even assuming a general relationship between strength and thickness. Thus, in any fundamental study, thickness should be measured as near as possible to the point of damage and, in addition, the membrane should first be removed. The relationship then applies to that area, but not necessarily to any other.

In passing, the even more remote and inaccurate assessments of strength, such as specific gravity and percentage shell should be condemned. They only assess mean thickness approximately and this alone is of little help even if assessed accurately.

A further point to bear in mind is that the measurement of strength which gives the best correlation with thickness is not necessarily the best measurement of strength. Thickness is really a red herring in this context. The problem is to find the method for measuring strength which correlates best with shell damage in the field, but the difficulty



here is that shells can break in so many ways and their strength in response to one kind of insult might be quite different from their strength in response to another kind. It might, therefore, be advantageous to use more than one method of measuring strength in order to find birds laying eggs which are "strong" in a number of different respects.

#### *Other factors associated with shell strength*

With all methods the shell is weakened by water and the translucent areas of the shell are weaker than the opaque areas. However, when the shell is thoroughly wet or thoroughly dry there is no difference in the strength of areas which were originally translucent or opaque. This suggests that translucent areas contain more water than opaque areas but it is not yet clear why this is so. It should, however, be stressed that shells with many translucent areas are not necessarily weaker than shells with few. The translucent areas on one shell may be stronger than the opaque areas on another (Tyler and Geake, 1964e).

From a number of studies, using cracking and crushing methods it is apparent that the membrane does not affect shell strength. But some recent results using the snapping method have shown that in general the shell is weaker when snapped outwards instead of inwards, that the cuticle gives added strength when the shell is snapped inwards and that the membrane gives added strength when the shell is snapped outwards.

#### *Types of shell damage*

One of the difficulties inherent in most methods of measuring strength, apart from the deformation method, is that the same method does not always produce the same type of shell damage. Thus with a flat plate the shell may crack cleanly or slowly crumble in concentric rings around a central point. Similarly a ball falling on one spot may produce a crack or push out a small round piece of shell.

Furthermore, shell sections reveal that incipient cracks may develop before any visible signs of damage can be seen even with the candling lamp. And even before this the calcite crystals of the shell may show twinning planes (Tyler and Moore, 1965).

#### *Acknowledgement*

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## PART IV

### ENVIRONMENTAL PHYSIOLOGY

# HEAT REGULATION AND ENERGY METABOLISM IN THE DOMESTIC FOWL

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## *Synopsis*

EXPERIMENTS WITH adult North Holland Blue hens have been performed to investigate the influence of environmental conditions such as temperature and humidity on the heat production, gaseous exchange, body temperature, water and food consumption and energy balance. A respiration chamber has been constructed for determining the energy metabolism of one and the same animal over periods of several months under conditions, which vary in temperature and humidity. The carbon dioxide production as well as the oxygen consumption could be recorded continuously, together with the body temperature of the experimental animal.

Kleiber's formula  $B.M.R. = 70 \times W^{3/4}$  for calculation of the basal metabolic rate in mammals also gave very satisfactory data in fowls. For practical purposes the ventilation required for any number of birds of any body weight may be calculated from their body weight. The rate of ventilation is independent of the cubic capacity of the poultry house and is only correlated with the number of birds and their body weight.

At temperatures below 20°C. heat production increases with falling temperature, whereas the evaporative heat loss could be estimated at about 20 to 30 per cent of total heat loss. Within this temperature range the food consumption also increases with a fall in temperature and the water intake is strictly correlated with food consumption.

At temperatures above 20°C. the heat production is maintained at a constant level, whereas the evaporative heat loss increases considerably. Physical heat regulation thus seems to be responsible for maintaining body temperature, although at temperatures over 34°C. the body temperature rises slightly over 42°C. in spite of an evaporative heat loss of up to as high as 80 per cent of the total heat produced.

Food consumption decreases rapidly at the higher temperatures and the water intake is no longer correlated with food consumption, but is mainly a function of evaporative heat loss.

The maintenance of a relatively low humidity in the poultry house is of major importance at high environmental temperatures, in order to prevent hyperthermia and an increased susceptibility to respiratory diseases. The ventilation should therefore be adapted to the water vapour content of the air, particularly during the hot seasons.

It could be assumed that the ventilation rate, required for maintaining a moderate humidity level would be greater than that for eliminating the amount of carbon dioxide produced. From data, determined for energy balance, body temperature, heat production and food consumption the most favourable environmental conditions for laying birds would correspond with a temperature between 20°C. and 30°C.; food consumption will be sufficient to keep the animals under maintenance and productive condition, total heat production will be at a minimum and the evaporative heat loss less than half the total heat production. The humidity of the air will be less critical than at higher environmental temperatures.

### *Introduction*

At the present time there is a growing interest among agricultural and veterinary scientists in problems associated with the housing of chickens, broilers and laying hens. The influence of environmental conditions such as temperature, humidity and the composition of the air on the production, on the one hand, and on the susceptibility to diseases on the other, justifies a scientific approach to all problems connected with ventilation and insulation of poultry houses and the mechanism of heat regulation in poultry of all kinds.

Though much attention has been paid, in several countries, to practical aspects it must be emphasised that the majority of the available data is based on the results of experimental work on poultry metabolism which has been performed with methods that are now obsolete. For an extensive review of the literature the reader should refer to the papers of Barott, Fritz, Pringle and Titus (1938); Kleiber and Dougherty (1933); Mitchell and Kelley (1933); Romijn (1950); Romijn and Lokhorst (1961, 1964); van Albada (1960); Borchert (1961); Osbaldiston and Sainsbury (1963); Hill (1951); Esmay (1962); Pero (1960); Reuter (1958); Gerriets, Werner and Stahl (1955).

Because of improvements in the methods of gas analysis and the availability of techniques for continuous recording of the oxygen and carbon dioxide content of air samples and of heat production, long-term experiments in metabolic research of high accuracy can now be carried out. Moreover, the construction of climatic chambers, supplied with adequate devices for controlling temperature and humidity, affords the opportunity of performing fundamental research on respiratory metabolism under different climatological conditions. The figures obtained for oxygen consumption, carbon dioxide production and heat loss of chickens as functions of body weight, nutritional status and

environmental flux must be the basis on which the ventilation and insulation of poultry houses can be calculated. From the nutritional standpoint, moreover, the calculation of total energy balance in laying birds and broilers under the above-mentioned conditions would assume greater importance.

### *Experimental*

The experiments to be described were performed with North Holland Blue fowls, using growing chickens as well as adult cocks and hens, having body weights of about 4 and 3 kg. respectively. Each animal was put into a respiration chamber (Fig. 1) of about 300 litres capacity; the temperature of which can be adjusted to any desired level from  $-10^{\circ}\text{C}$ . upwards and the humidity controlled by means of a hygrostat. Ventilation is maintained by a pump (P) with a variable capacity, adjusted to the animals' size in order to provide a suitable excess of  $\text{CO}_2$  and  $\text{O}_2$  deficit in the air breathed (the "respiration air"). The incoming outdoor air (O.A.) and the respiration air (R.A.),

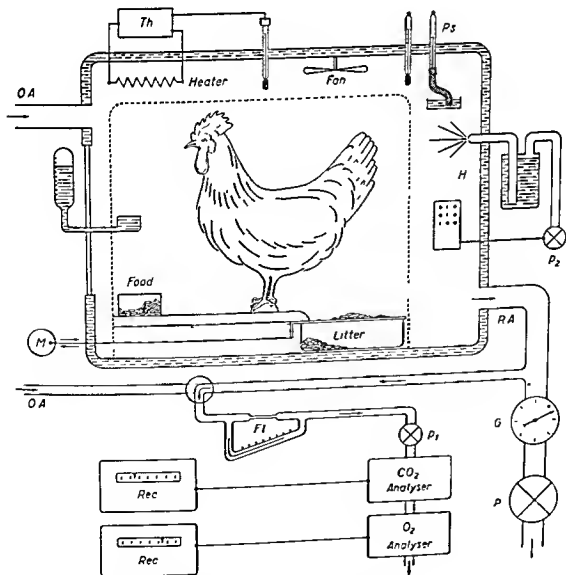


FIG. 1. Respiration chamber for metabolic research at different temperatures and levels of humidity.

are sampled by a small pump ( $p_1$ ) and flow through a gas analyser based on the heat conductive principle (diaferometer). The output of the analyser is, after suitable amplification, continuously recorded on a mV-recorder of the continuous balance type. Each division on the paper corresponds with an increase of 0.01 per cent  $\text{CO}_2$  and a decrease of 0.01 per cent  $\text{O}_2$ .

Total heat production may be calculated from  $\text{O}_2$  consumption and  $\text{CO}_2$  production with sufficient accuracy, according to the formula given by Romijn and Lokhorst (1961a), and which is based on the figures of Carpenter (1948) for combustion of carbohydrates and fat and those of King (1957) for the metabolism of protein in birds:

$$T = 3.871 \text{ O}_2 + 1.194 \text{ CO}_2 - 0.048 \text{ P}$$

where  $T$ =kcal.;  $\text{O}_2$ =oxygen uptake (litres);  $\text{CO}_2$ =carbon dioxide production (litres) and  $\text{P}=6.25 \times$  urinary nitrogen (g.).

The animals were fed with an "all mash" diet (pellets) with a productive energy of about 1.903 kcal./kg., 17 per cent crude protein, 89 per cent dry matter and a gross energy of 4.350 kcal./g. dry substance. Food and water were provided *ad libitum* and the quantities consumed were estimated by daily weighing of the containers. The droppings were automatically collected in a closed container and analysed for total nitrogen, urinary nitrogen (Ekman, 1948), as well as for water content and energy (bomb calorimeter). Artificial light was provided for 12 hr. during a 24 hr. period. A thermocouple was inserted into the subcutaneous tissues under the left wing and the body temperature recorded continuously.

The respiration chamber was opened once daily for about 10 min. in order to weigh the animal, the food and the excreta. Owing to the relatively high ventilation rate the gaseous equilibrium in the climatic chamber was restored after  $3.91 \times \frac{V}{a}$  min., where  $V$  is the

volume of the respiration chamber in litres and  $a$  is the ventilation rate in litres per minute. The reason for discarding the protein metabolism in the calculation of the total heat production is evident in Table 1, in which the heat production of an incubating egg, of an adult starving fowl as well as an adult bird on a normal diet has been calculated according to the complete formula (1) and a simplified formula.

From the results given in Table 1 it may be concluded that in spite of the important contribution of protein combustion to the total heat expenditure of the adult animal under normal nutritional conditions, the laborious determination of urinary nitrogen is not essential in order to obtain correct figures for total heat production. To estimate the energy balance it is quite sufficient, therefore, to determine the calorific value of the food and the mixed excreta, in addition to the gaseous exchange of the animal.

The results obtained with 12 North Holland Blues, 7 Cape ducks

and 1 Pekin duck are in very close agreement. The data obtained with hen No. 3 have been plotted in Fig. 2, which shows striking daily variations in metabolic activity and which are obviously correlated with the time of feeding. Oxygen consumption as well as  $\text{CO}_2$  production are at a minimal level early in the morning just before the artificial lighting starts at 7 a.m. In hen 3 (Fig. 2) the  $\text{O}_2$  consumption during this period amounts to 4.60, 4.78 and 4.30 l./2 hr., with a corresponding  $\text{CO}_2$  production of 2.85, 2.97 and 2.95 l./2 hr. under conditions of normal feeding.

TABLE 1

*O<sub>2</sub> consumption, CO<sub>2</sub> production as well as total heat production under different nutritional conditions*

<i>Chicken embryo</i>	
O <sub>2</sub> consumption over 21 days	6.225 l.
CO <sub>2</sub> production over 21 days	4.532 l.
R.Q. = 0.73	
Metabolised protein 0.528 g. = 2.218 kcal.	
Heat production (complete formula)	29.483 kcal.
Heat production (short formula)	29.508 kcal.
Difference = 0.025 kcal. = 0.1 per cent of total heat production.	
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<i>Adult fowl (fully fed)</i>	
O <sub>2</sub> consumption over 24 hr.	53.088 l.
CO <sub>2</sub> production over 24 hr.	45.125 l.
R.Q. = 0.85	
Metabolised protein 9.74 g. = 40.9 kcal.	
Heat production (complete formula)	258.91 kcal.
Heat production (short formula)	259.38 kcal.
Difference = 0.47 kcal. = 0.18 per cent of total heat production.	
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<i>Adult fowl (starving)</i>	
O <sub>2</sub> consumption over 24 hr.	42.470 l.
CO <sub>2</sub> production over 24 hr.	29.198 l.
R.Q. = 0.69	
Metabolised protein 3.62 g. = 15.2 kcal.	
Heat production (complete formula)	199.09 kcal.
Heat production (short formula)	199.26 kcal.
Difference = 0.17 kcal. = 0.1 per cent of total heat production.	

As soon as the artificial illumination is switched on, there is an immediate increase in metabolism although feeding does not take place until 2 hr. later. Feeding results in a further rapid increase of respiratory metabolism which reaches a maximum early in the afternoon and is due to the greater activity of the digestive tract and the specific dynamic action of the food. The  $\text{O}_2$  consumption increases to 6.93 and 6.66 l., whereas the  $\text{CO}_2$  production is 5.31 and 5.56 l. 2 hr., respectively. The respiratory quotient is 0.62 and 0.69 early in the morning and 0.77 and 0.83 early in the afternoon. Metabolic activity increases therefore by 56 and 51.5 per cent within a 5 hr. period.

A second point of interest is the change in the R.Q. over a 24 hr. period. Even after an interval of 22 hr. the R.Q. is lower than



could be expected when pure fat is being combusted whereas at the time of maximal gas exchange the R.Q. corresponds with a figure associated with "mixed" combustion. Obviously, in the fowl, the basal metabolic state when food is withheld is realised much earlier than in mammals. The problem of the extremely low figure of the R.Q. has been discussed by Romijn and Lokhorst (1964). The aforementioned situation is characteristic of animals fed once daily and eating their food rapidly, as a rule within 1 or 2 hr.

When food is withheld the metabolic activity decreases further and 2 days after the last feed, an  $O_2$  consumption of 3.43 l./2 hr. and a  $CO_2$  production of 1.94 l./2 hr. was recorded at 7 a.m.. This is the "basal metabolic rate", since longer fasting causes no further fall. In the fasting animal, daily fluctuations in metabolic intensity are similar to those recorded in the normal animal, but the increase during daytime is very limited because food is withheld.

Oxygen consumption increases from 3.43 to 3.89 l./2 hr. during the third day of starving; from 3.45 to 4.16 on the fourth and from 3.40 to 3.46 on the fifth day, corresponding with increases of 14.0,

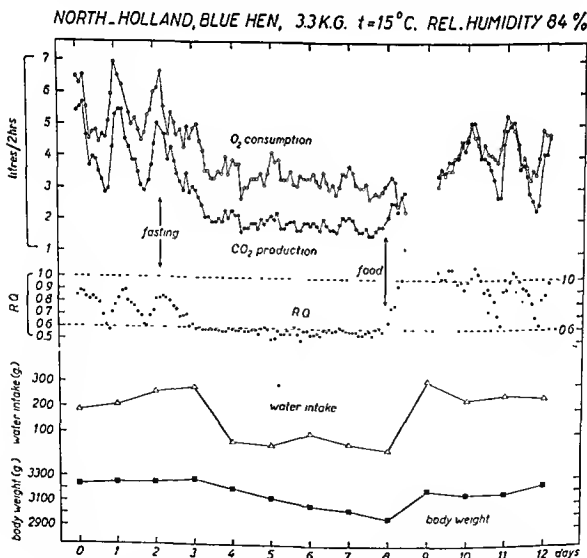


FIG. 2. Daily fluctuations in metabolic activity of a North Holland Blue hen, No. 3.

18.4 and 3.4 per cent of the lowest figure at 7 a.m. In the normal animal, therefore, the increase of metabolic activity during daytime is mainly caused by food intake and absorption, rather than by muscular exercise resulting from the artificial illumination. In the starving animal on the other hand, the daily variations in metabolism are not accompanied by changes in the R.Q. as is the case in the normally fed fowl; on the contrary, the R.Q. was 0.69, 0.57, 0.59 at 7 a.m. on successive days of starving, remaining constant on subsequent days. Occasionally, the very low figure of 0.52 was obtained. Under starving conditions the total respiratory metabolism is, of course, considerably lower than in the normal animal. For example in hen No. 3 (Fig. 2) the heat production, under conditions of daily feeding, amounts to 94.8 kcal./kg./day at 10°C. and is only 59.7 kcal./kg./day during starving, which represents a decrease of 36.8 per cent.

One of the most striking features observed during starving is the low respiratory quotient. Noteworthy also is the fact that the R.Q. remains low during the starving period, until feeding is resumed. Thereafter, not only does the respiratory metabolism increase rapidly, but the R.Q. also rises sharply, and may even attain a value of 1.24, a figure which is higher than might be expected when carbohydrates are metabolised. Three days after feeding is resumed normal conditions are restored with daily fluctuations in metabolic activity and R.Q.

#### *The heat increment of food*

It is well known that the intake of food and its accompanying digestion and absorption results in a considerable rise in the metabolic rate, and that this increase of heat production (heat dissipation) decreases the amount of expected productive energy and should be subtracted from the metabolisable energy. As a matter of fact, heat production, even under conditions of maintenance, is considerably higher than in the fasting animal. This difference, the "heat increment of food", has been determined in adult fowls by performing metabolic experiments under conditions of normal feeding (maintenance) and after a starving period of 4 days. Recent investigations of Romijn and Lokhorst (1964) on the metabolism of starving fowls and ducks have shown that after 2 days the metabolic rate reaches its basal level and the animal is under "basal" or "standard" conditions. The results, obtained with several hens and cocks agree very closely and are illustrated in Fig. 3.

From Fig. 3 it may be concluded that in the starving hen, heat production remains extremely constant over a 24-hr. period and the respiratory quotient is correspondingly low. The total heat production of the starving hen was shown to be of the order of 180 kcal. day, corresponding with a consumption of 38.3 l. and a CO<sub>2</sub> production of 27.3 l. For a full discussion of the R.Q. when food is withheld, the reader should consult the paper by Romijn and Lokhorst (1964).

Under maintenance conditions heat production amounts to 286 kcal./day and is, therefore, 106 kcal./day or 59 per cent higher than during starving. Moreover, the daily fluctuations in heat production become very pronounced; switching on the light and supplying food produce a considerable increase in the metabolic rate. The R.Q. in the normally fed animal is higher than in the starving animal and attains a minimum just before feeding. Thereafter, as a consequence of the mixed combustion of carbohydrates, fats and protein the R.Q. rises rapidly.

The total oxygen consumption of the animal under maintenance conditions amounted to 59.6 l./day while carbon dioxide production amounted to 47.7 l. during the same period. The rise in oxygen consumption is therefore 56 per cent of the basal consumption, whereas the increase in carbon dioxide production, owing to the alteration of the respiratory quotient which accompanies the intake of food, is not less than 75 per cent of the standard production. In terms of "ventilation", therefore, carbon dioxide production, because it is so obviously increased and influenced by food consumption, assumes a role of major importance.

Moreover, because of the sensitivity of the respiratory centre to changes in the partial pressure of carbon dioxide, the physiological

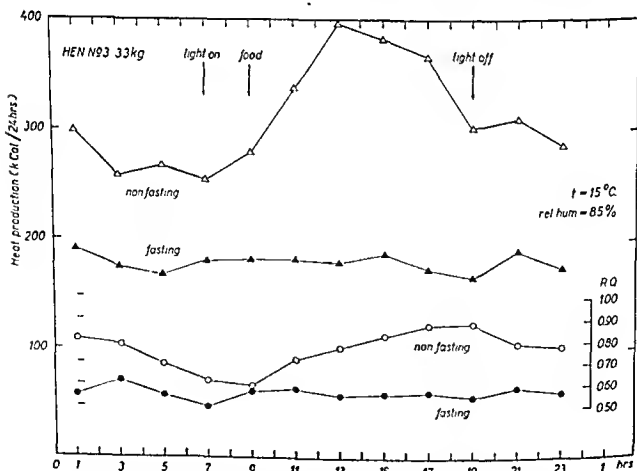


FIG. 3. Heat increment of food in the North Holland Blue adult hen, No. 3, 3.3 kg.;  $t=15^{\circ}\text{C}$ ., Food intake=140 g./day; water consumption 216 g./day.

effects of this gas on the respiratory activity of the animal is more pronounced than with a corresponding reduction in the percentage of oxygen. On the other hand, owing to the efficiency of pulmonary ventilation and the shape of the oxygen dissociation curve of chicken blood, even a fall of several per cent in the oxygen content of the blood does not reduce the amount of oxygen to dangerous limits. The calculation of the ventilation of a poultry house with a flock of 1,000 birds under maintenance conditions will serve as an example.

In spite of the fact that pulmonary ventilation rate does not increase until the carbon dioxide concentration exceeds 1.48 per cent (Romijn, 1950), the carbon dioxide content of the inspired air should, preferably, not rise above 0.5 per cent and the minimum quantity of air required for ventilation should, therefore, be such as would ensure that this concentration of the gas ( $\text{CO}_2$ ) is present in the air leaving the poultry house.

One bird produces 47.7 l.  $\text{CO}_2$ /day and 1,000 birds 47.7  $\text{m}^3$ .  $\text{CO}_2$ . Elimination of 47.7  $\text{m}^3$ .  $\text{CO}_2$  with air containing 0.5 per cent of  $\text{CO}_2$  requires therefore, that the poultry house is ventilated with  $200 \times 47.7 = 9,540 \text{ m}^3$ . fresh air/day or 6.63  $\text{m}^3$ ./min. With an oxygen consumption of 59.6  $\text{m}^3$ ./day the reduction in the percentage of oxygen in the ventilation air is of the order of 0.62 per cent. Thus, as a result of the gaseous exchange of the birds and the rate of ventilating the poultry house a situation is achieved in which the air breathed by the birds has a composition of 0.5 per cent carbon dioxide and 20.32 per cent of oxygen, which is very reasonable.

It should be mentioned, of course, that under practical conditions it may be necessary to ventilate at a higher rate, especially in cases of accumulation of moisture, particularly at relatively high temperatures. The influence of temperature and humidity on the physiological condition of the birds will be discussed below.

In any event, an estimate of the minimum rate of ventilation in the construction of poultry houses merits consideration in order to be quite sure that the capacity of the fans together with the natural ventilation of the building does not fall below the minimum. Needless to say, in practice it will be impossible, in most cases, to determine the respiratory metabolism of the birds, yet for practical purposes it is very important to get a rough idea about the carbon dioxide production of the total population. In practice the only figure which can be estimated with sufficient accuracy is the body weight of the birds and it would be of great advantage therefore to express the metabolic rate in terms of body weight. In the case of the basal metabolic rate this may be estimated with surprising accuracy. It is clear that a heavy bird has a greater basal metabolism than a light one, but the correlation is not a linear one.

The surface area of the body would give a better linearity but in mammals, according to Kleiber (1961), the correlation between basal

metabolic rate and the  $\frac{3}{4}$  power of body weight approximates almost perfect linearity, according to the formula:

$$\text{B.M.R.} = 70 \times W^{\frac{3}{4}}$$

where: B.M.R. is the basal metabolic rate (kcal./day) and  $W$  is the body weight (kg.).

Using Kleiber's formula, in the starving bird (Hen No. 3, Fig. 2) with a body weight of 3.3 kg., heat production was found to be 172 kcal./day, whereas a figure of 180 kcal. was obtained by indirect

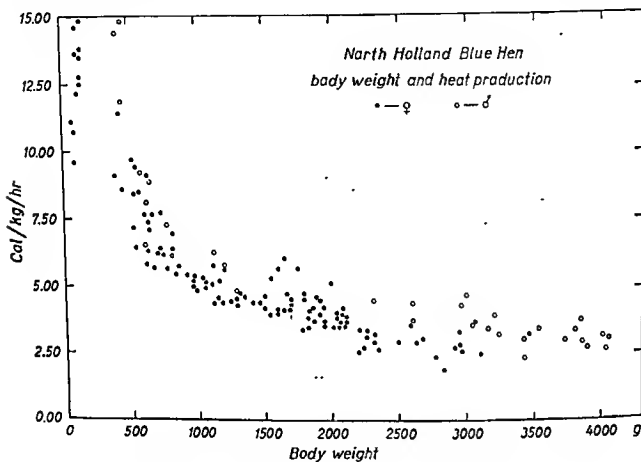


Fig. 4. Body weight and heat production in the starving North Holland Blue fowl.

calorimetry. The close agreement between these two figures justifies the acceptance of the formula for birds as well as mammals.

Using the basal metabolic rate, carbon dioxide production can be calculated by dividing this figure by 6.6, which is the calorific equivalent of 1 l. of  $\text{CO}_2$  in the starving state. Under conditions of maintenance or production the quotient should be multiplied by 1.75 and the result gives the total carbon dioxide production of a bird with a body weight of 11 kg. Finally, multiplying the total number of birds of the same body weight by a factor of 200 gives the required ventilation necessary to keep the carbon dioxide content in the poultry house at 0.5 volumes per cent.

To give an example: In a poultry house containing 1,000 chickens, each with a body weight of 1 kg.

$$\text{B.M.R.} = 70 \times 1^{\frac{3}{4}} = 70 \text{ kcal./day}$$

CO<sub>2</sub> production amounts to  $\frac{70}{6.6} \times 1.75 = 18.56$  l./day and required minimal ventilation:  $200 \times 1,000 \times 18.56 = 3,712$  m<sup>3</sup>./day or 2.58 m<sup>3</sup>./min. It should be emphasised that this ventilation is independent of the cubic capacity of the poultry house and is correlated solely with the number of animals. The volume of the house does, however, influence the time which must elapse before which equilibrium is established between elimination of carbon dioxide and its production by the animals. The smaller the poultry house, the sooner will equilibrium be reached and the time of equilibration may be expressed by the formula:

$$t \text{ (min.)} = 3.91 \times \frac{V}{a} \text{ (Noyons, 1937),}$$

where  $V$  is the total cubic capacity of the poultry house (m.<sup>3</sup>) and  $a$  is the ventilation in m<sup>3</sup>./min.

Consequently in the foregoing example with 1,000 chickens of 1 kg. body weight, if they are housed in a cubic space of 516 m<sup>3</sup>., the gaseous equilibrium will be established after

$$3.91 \times \frac{516}{2.58} = 782 \text{ min. (13 hr.)}$$

### *Temperature and humidity*

Chickens are homoiothermic animals and have the ability therefore to maintain their body temperatures of 41-42°C. over a wide range of environmental temperatures. In Fig. 5 the influence of environmental temperature on body temperature has been plotted. From a consideration of this figure, it may be concluded that the animal, under normal nutritional conditions, is able to maintain its body temperature within the range 41-42°C. up to an environmental air temperature of about 34°C. With a rise of environmental temperature, up to about 20°C., heat production decreases gradually in accordance with the mechanism of chemical heat regulation. Heat dissipation within this range is mainly achieved by radiation and convection, whereas the evaporative heat loss is relatively constant at a level of 20 to 30 per cent of the total heat loss. As soon as the environmental temperature rises above 20°C. heat production is maintained at a constant level and the animal is then in the zone of thermal neutrality.

Under these conditions the excess heat is increasingly eliminated by evaporation and at a temperature of 34°C., at least 50 per cent of the total heat loss is achieved by the physical mechanism of evaporation. The critical temperature of the adult chicken under conditions of normal nutrition is therefore 20°C.; at higher environmental temperatures the heat production and heat loss remain constant and the higher the temperature, the more heat is lost by evaporation. From a

physiological standpoint the increase in evaporative heat loss is not very economic owing to the fact that evaporation in the fowl is exclusively a respiratory phenomenon. Increase in evaporation requires therefore a corresponding increase in respiratory activity, which is associated with a rise in the metabolic activity. In this respect the humidity of the inspired air will greatly influence the amount of evaporative water loss and this condition will be of increasing importance, the higher the environmental temperature. The capacity of the air to contain water vapour varies widely with the temperature (Table 2).

The temperature of the expired air is slightly lower than the body temperature and in the fowl will be about  $40^{\circ}\text{C}$ . At the same time it will be practically saturated with water vapour and as may be seen from Table 2, it will contain  $51\text{ g. water/m}^3$ . At an environmental temperature of  $20^{\circ}\text{C}$ . and a relative humidity of 100 per cent the evaporative water loss of the chicken will be, therefore,  $51 - 17.1 = 33.9\text{ g. water/m}^3$  respired air. With a high environmental temperature of about  $34^{\circ}\text{C}$ . inside the poultry house and a relative humidity of 100 per cent, the water loss per  $\text{m}^3$  of expired air will be as low as  $51 - 37.2 = 13.8\text{ g.}$  An evaporative heat loss of  $58\text{ kcal./day}$  for example would correspond with a respiratory water loss of  $100\text{ g.}$  ( $0.58\text{ kcal./g.}$  of water). At an environmental temperature of  $20^{\circ}\text{C}$ . and full saturation, this would correspond with a lung ventilation of  $\frac{100}{33.9}$  or about  $3\text{ m}^3/\text{day}$ . At a temperature of  $34^{\circ}\text{C}$ . and full

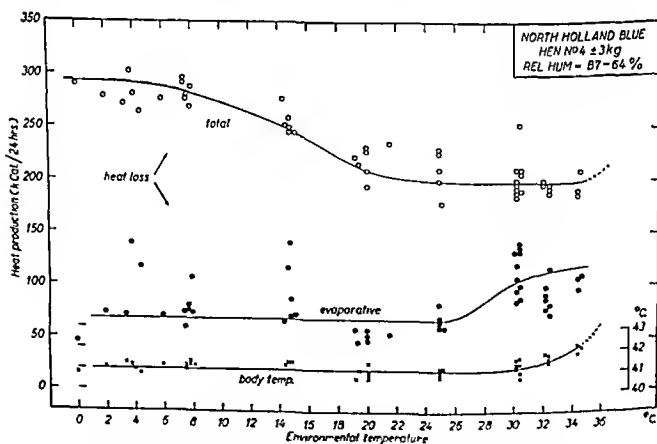


FIG. 5. The influence of environmental temperature on body temperature and heat loss in a North Holland Blue laying hen.

saturation, the evaporation of 100 g. of water would require a pulmonary ventilation rate of at least  $\frac{100}{13.8}$  or about 7 m<sup>3</sup>./day.

In hen No. 4 (Fig. 5) the heat production at 20°C. as well as at 34°C. amounts to 210 kcal./day. At 20°C. about 52 kcal. are lost by evaporation at a relative humidity of 87 per cent, corresponding with 89 g. of evaporated water; 1 m<sup>3</sup>. expired air contained 51 g. of water vapour and 1 m<sup>3</sup>. inspired air  $\frac{87}{100} \times 17.1$  or 14.9 g. of water.

Evaporation of 89 g. of water thus requires a ventilation of the respiratory tract with  $\frac{89}{36.1}$  or about 2.4 m<sup>3</sup>./day.

TABLE 2

*Mass of water vapour in saturated air*

Temp. °C.	Mass of water in g./m <sup>3</sup> .
0	4.8
10	9.3
20	17.1
30	30.0
34	37.2
40	51.0

At 34°C. not less than 50 per cent of total heat or 105 kcal. is lost by evaporation corresponding with an evaporation of 190 g. of water. As the relative humidity was 64 per cent at 34°C., 1 m<sup>3</sup>. of inspired air contained 23.8 g. of water vapour. The corresponding pulmonary ventilation rate may be estimated as  $\frac{190}{27.2}$  or 7 m<sup>3</sup>./day.

To prevent an augmentation in the water vapour content of the surrounding air it is necessary, therefore, to provide a ventilation rate which is adjusted to the sum total of water elimination of the animals, on the one hand, and to other evaporating sources on the other. In the case of a poultry house with a population of 1,000 adult hens as mentioned above, a ventilation rate of 9,540 m<sup>3</sup>. day was suggested in order to keep the CO<sub>2</sub> percentage on or below 0.5 per cent.



ventilation, in order to prevent a rise in humidity over 87 per cent. The requisite ventilation may then be expressed as  $\frac{177,700}{14.9}$  or 11,880  $\text{m}^3/24 \text{ hr.}$  or  $8.25 \text{ m}^3/\text{min.}$  The evaporative weight loss of the birds, even at moderate temperatures, rather than their carbon dioxide production and oxygen consumption is, therefore, the determining factor in the adjustment of ventilation. The higher the environmental temperature, the greater the water vapour produced, and hence the greater the ventilation that is required to maintain a moderate humidity.

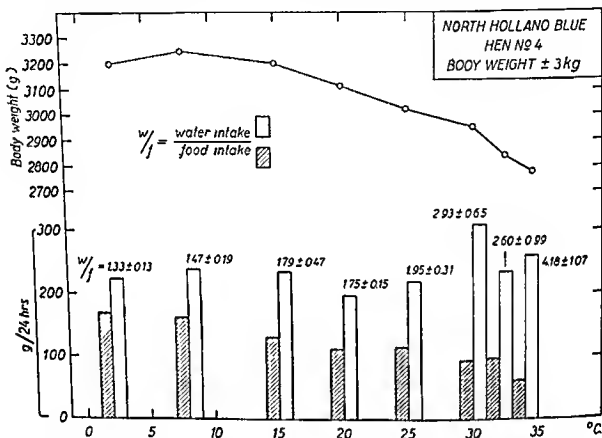


FIG. 6. The contribution of evaporative heat loss to the total heat loss in a North Holland Blue laying hen, No. 4. Body weight  $\pm 3 \text{ kg.}$

The important contribution of evaporative heat loss to the total heat loss is well illustrated in Fig. 5. At environmental temperatures from  $30^\circ\text{C.}$  up to  $34^\circ\text{C.}$ , half of the total heat dissipated is given off as evaporative heat, resulting in an increased water consumption as is illustrated in Fig. 6 for hen No. 4.

Food consumption decreases with rising environmental temperatures, whereas water intake remains approximately constant up to  $25^\circ\text{C.}$  With a rise in air temperature above  $25^\circ\text{C.}$ , a further rise in water consumption can be observed. At  $20^\circ\text{C.}$ , the water intake is about 1.75 times the food consumption, whereas, at  $34^\circ\text{C.}$ , there is a fourfold increase. The decrease in food consumption at higher environmental temperatures is striking: heat production remaining constant (Fig. 5) there will be eventually a marked decrease of the nett energy and eventually a considerable loss in body weight.

The figures, obtained for the energy balance of the same animal (hen No. 4) have been plotted in Fig. 7.

The decrease in gross energy at the higher environmental temperatures is accompanied by a decrease in metabolisable energy and, as a result of the constant heat production, the nett energy becomes negative at a temperature of 34°C. In spite of the fact that egg production was maintained at temperatures of 30°C. and higher, over a period of several days, it is clear that these environmental conditions should be avoided as far as possible. At environmental temperatures of 30°C. and above, the body temperature rises gradually and at 34°C. a slight hyperthermia has developed with a body temperature somewhat higher than 42°C. It should be emphasised that under these conditions the relative humidity of the air is decisive in determining the survival or death of the bird.

In starving North Holland Blues, Romijn and Lokhorst (1961*b*) found hens to be more susceptible to heat than cocks. Exposure of hens to a temperature of 35°C. at a relative humidity of 95 per cent over a 4 hr. period resulted in a rise of body temperature from 41°C. to 44°C. and a corresponding increase in metabolic rate. At a humidity level of 36 per cent, however, the same temperature has a far less detrimental effect and a much longer period of exposure can be tolerated before a fatal body temperature is developed. During the bird's exposure to 37°C. and a relative humidity of 30 per cent, the evaporative heat loss was more than 50 per cent and occasionally as high as 84.3

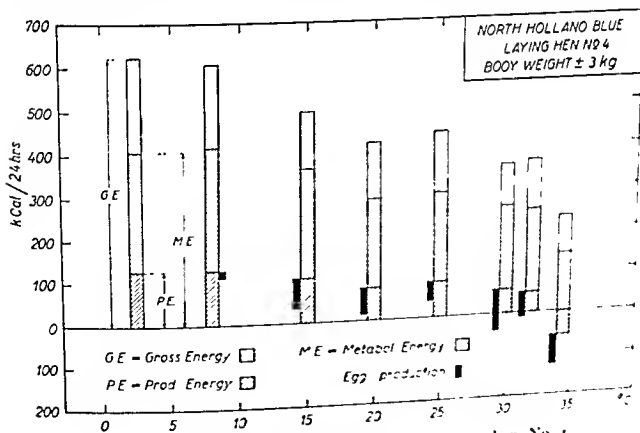


Fig. 7. Energy balance of a North Holland Blue laying hen, No. 4  
Body weight  $\pm 3$  kg

per cent of the total heat loss. In consequence of the heat increment of food it may be anticipated that fowls under normal nutritional conditions have a smaller heat tolerance than starving animals.

In Table 3 the heat tolerance of an animal under normal nutritional conditions has been determined. As is clearly shown the humidity of the environmental air is the determining factor in maintaining body temperature as soon as the air temperature rises above 33°C.

TABLE 3  
*Heat tolerance of a North Holland Blue hen*  
*Body weight 3.4 kg*

Day No.	Temp. °C.	Relative humidity per cent.	Body temp. °C.	Heat production kcal./day.	Evaporative heat loss kcal./day.	Evaporative heat loss per cent of total heat loss
1	24.2	78	40.8	187.7	51.8	27.6
2	24.2	80	40.7	182.8	50.6	27.7
3	24.1	84	40.7	183.5	39.7	21.6
4	24.5	40	40.5	170.1	86.3	50.7
5	24.2	40	40.6	174.3	87.4	50.2
6	33.7	40	41.3	176.9	115.0	65.0
7	33.8	40	41.4	180.0	149.5	80.3
8	33.8	90	42.3	196.0	75.9	38.7

Not less than 149.5 kcal. or 80.3 per cent of total heat is lost by evaporation and it is therefore very essential for the wellbeing of the animal to keep the humidity as low as possible. Forced ventilation of the poultry house may be helpful, but it is not always successful where the humidity of the outdoor air is high and, in this event, decreased productivity and increased susceptibility to disease or even death from heat collapse may be the final outcome.

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# THE RESPONSE OF THE IMMATURE CHICKEN TO AMBIENT TEMPERATURE

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## *Synopsis*

DURING THE brooding period, the response of a population and an individual chicken maintained singly, is different. The individual increases its food consumption as the temperature of the environment cools—the range was  $32.2^{\circ}\text{C}.$  to  $7.2^{\circ}\text{C}.$ —but the energy lost as heat is so excessive that the growth rate at  $7.2^{\circ}\text{C}.$ , is significantly inferior to that at  $32.2^{\circ}\text{C}.$  The population responds to suboptimal temperatures not by increasing its food intake but by decreasing its heat loss. The individual birds in a population huddle into groups and as the temperature becomes more unfavourable the group packs more closely. The population employs its behavioural reaction which maintains its food conversion efficiency at the expense of growth rate, whereas the food conversion efficiency of the bird maintained singly, becomes worse as the temperature becomes less favourable.

The suboptimal temperature stimulates the food intake of the individually reared bird whereas it primarily stimulates a behavioural reaction within a population.

## *Introduction*

At hatching, the chicken is relatively mature in its thermal independence to the environment. The body temperature of the chicken a few hours after hatching is  $38.9^{\circ}\text{C}.$  Hemsley (personal communication) has found that as chickens are taken off the incubator trays, body temperature may be as low as  $36.7^{\circ}\text{C}.$  The chicken's locomotor activity is already highly developed at hatching and it can maintain its blood glucose level for several days after hatching even without food, and although its liver and heart glycogen content is low (Beattie, 1964). Its body temperature rises to  $41.7^{\circ}\text{C}.$  within the first fortnight after hatching which suggests that its rate of development of thermoregulation is slow; this rate of development of thermoregulation is not as temperature dependent as might be expected because chickens reared individually in a cold environment ( $7.2^{\circ}\text{C}.$ ) took only 2 days

longer to build up their body temperature to  $41.7^{\circ}\text{C}$ . than those reared at  $32.2^{\circ}\text{C}$ .

The behaviour pattern of the newly hatched chicken is influenced by ambient temperature. In a warm environment ( $32.2^{\circ}\text{C}$ .) the chicken is very active and has short rest and sleeping periods; as the environment cools, the activity is less and in a cold environment ( $15.6^{\circ}$ - $12.8^{\circ}\text{C}$ .) the chicken spends most of its time squatting with its feathers flattened or ruffled. This behaviour suggests that the chicken in the cool environment has an instinctive way of heat conservation. With a group of chickens, this behavioural response to a cooling environment leads to their packing together to form a dense carpet. Huddling was termed social temperature regulation by Brody (1945).

The outstanding landmark in the literature discussing the effect of environmental temperature on the metabolic heat response of the chicken is the paper of Barott and Pringle (1946). This shows that the metabolic response of the individual chicken to the environmental temperature is already well developed at hatching but it has relatively limited ability of heat conservation. The environmental temperature range for minimal metabolic rate is around  $35^{\circ}\text{C}$ . at hatching and is clearly defined; as the chicken grows, progressively cooler temperatures are associated with the minimal metabolic rate. Mount (1962) has shown that piglets by huddling together can modify the influence of the environment to such an extent that the increase in the metabolic response per unit mass of active tissue in cold surroundings is much less than with single pigs. There is no published work of the response of a group of chickens.

The conventional husbandry recommendations for brooding small groups of chickens is an ambient temperature of  $35^{\circ}\text{C}$ . for the first few days after hatching gradually reducing to  $18.3^{\circ}\text{C}$ . at 6 weeks of age. Osbaldiston and Sainsbury (1963) found a constant ambient temperature of  $21.1^{\circ}\text{C}$ . to be associated with the fastest average growth rate of populations of approximately 1,000 chickens. The aim of this discussion is to show the dissimilarity in the response of the chicken maintained singly, and the average individual of a population to a cooling environment.

### *Results and Discussion*

#### *The Influence of Air Temperature on the Average Growth Response of a Population*

of 39 g. was chosen as this was the averaging weight for these chickens a few hours after being taken from the incubator trays. It is clear that the chicken shows a marked response to air temperature, and in this case the response was significant ( $P < 0.05$ ) at 2 weeks of age between those populations reared at  $21.1^{\circ}$  and  $23.9^{\circ}\text{C}$ . and those reared at  $12.8^{\circ}\text{C}$ . The instantaneous growth rate  $k^*$  declines rapidly during

$$*k = \frac{\log_e W_2 - \log_e W_1}{T_2 - T_1}$$

the first few weeks of the brooding period and the differences in the 3 weeks' body weights resulting from the different growth rates, could be attributed solely to the air temperature. After the third week, the instantaneous growth rate in each climate was similar and the relative

TABLE 1

*The calculated average liveweight (grams) of populations (mixed sexes) at various ages, reared at various constant ambient temperatures if hatching at an average weight of 39 g.*

Age weeks	$26.7^{\circ}\text{C}$ .	$23.3^{\circ}\text{C}$ .	$21.1^{\circ}\text{C}$ .	$18.3^{\circ}\text{C}$ .	$15.6^{\circ}\text{C}$ .	$12.8^{\circ}\text{C}$ .
3	315	332	342	301	283	271
6	885	929	982	916	892	823
9	1,576	1,652	1,742	1,678	1,580	1,478

differences between the cumulative growth rates lessened. This similarity in the value of  $k$  from 3 to 9 weeks of age suggests that climate was not influencing the growth rate. Temperature exerted its effect only in early life; within the temperature range  $12.8^{\circ}$  to  $26.7^{\circ}\text{C}$ ., the growth rate became less sensitive to climate as the birds aged, body size becoming the most important factor in determining the succeeding weight gain after 3 weeks of age.

#### *The Response of the Individual Chicken in a Population to Ambient Temperature*

Although the temperature was able to alter the growth response of the population, Table 2 shows that the individual chicken of these populations showed a marked refractoriness in its response to the environment.

The response of the individual may be shown by the coefficient of variation of the individual weights about the mean. In an optimum climate it is to be expected that the relative deviation (coefficient of variation—mean weight) would be least and in the less favourable climates the relative deviation would increase. Chance (1957), Ashloub, Biggers, McLaren and Michie (1958), and Zucker, Atkinson, Schnell and Donovan (1958) have all agreed that when birds are placed in an unfavourable environment the variation of the body weights increases and that this population response characterises the response of the individuals comprising the population. Bird and

Gutteridge (1934) suggested that the females were more severely affected than males by adverse conditions.

The relative deviations of populations at 3 weeks and 6 weeks after hatching are very similar to relative deviations of the 9-week birds in Table 2. The relative deviation at hatching is slightly less. Thus after hatching there is a small response but after 3 weeks of age there is no apparent response. This refractoriness is attributable to the hybrid genotype of this strain of chicken. Presumably there is sufficient genetic variability to cope with the effects of this range of climate. Lerner

TABLE 2

*Effect of hatching weight on relative deviation of liveweights at 9 weeks*

Chickens from populations which were reared in a standardised environment were subdivided at the end of the experiment, on the basis of hatching weight and the relative deviation of the liveweight of the individuals in each group was calculated. Basis of subdivision was (a) Heavy hatching weight 42, 43 and 44 g., (b) Medium hatching weight 38, 39 and 40 g. and (c) Light hatching weight 34, 35 and 36 g.

(a) MALES	Hatching weight		
	Heavy	Medium	Light
Season of year			
Summer	7.2	7.8	—
Autumn	9.1	8.1	8.1
Winter	6.9	7.9	12.3
Spring	10.9	8.3	8.1
(b) FEMALES			
Summer	7.6	7.1	—
Autumn	6.2	9.3	7.8
Winter	9.7	7.0	9.7
Spring	9.2	8.2	8.2

(1954) with his concept of genetic and developmental homeostasis suggests that heterozygous populations such as between two inbred lines, would be more resistant to changes in the general environment, that is there would be less response to the environment. The chickens in Table 2, which were a commercial broiler chicken, behave according to Lerner's hypothesis. This refractoriness towards variation is of tremendous commercial importance, because with the methods of mass selection used to determine parental generations, the sole criteria which need to be considered for growth response is average liveweight of a population.

*The Influence of Ambient Temperature on the Individual Chicken maintained singly*

Table 1 shows that 21.1°C. is the optimum temperature for a population if growth is the criterion. When efficiency of energy utilisation is the criterion, Kleiber and Dougherty (1934) and Barott and Pringle (1946) have shown that 35.8° to 37.8°C. is the optimum temperature for young chickens. Table 3 is essentially in agreement



with the conclusions of Barott and Pringle. In this experiment the chickens were reared in constant temperatures of 32.2°, 23.9°, 15.6° and 7.2°C.

TABLE 3

*The age at which individual chickens maintained singly, in constant ambient temperatures, weighed 1,000 g. liveweight*

32.2°C.	23.9°C.	15.6°C.	7.2°C.
47 days	59 days	64 days	after 42 days the chickens averaged 250 g.

*Comparison between the Individual maintained singly, and the Population Response to Ambient Temperatures*

In both cases, the optimum temperature for growth did not fall as the birds grew and the effect of temperature was most pronounced in the first few weeks after hatching, except in the population studies 23.9°C. was associated with heavier liveweight gains in the first fortnight after hatching and at 3 weeks, 21.1°C. became associated with the heaviest gains. After 400 g. liveweight, body size was the most

TABLE 4

*The relative rate of growth  $\left(100 \frac{\Delta W}{W}\right)$  during the first 3 weeks after hatching for birds reared singly or in populations, at diverse brooding temperatures*

		Brooding temperatures					
		32.2°C.	23.9°C.	15.6°C.	7.2°C.		
A. Singly reared	Age after hatching						
	1 week	144	112	81	53		
	2 weeks	96	92	77	36		
	3 weeks	86	90	56	39		
B. Populations							
		26.7°C.	23.9°C.	21.1°C.	18.3°C.	15.6°C.	12.8°C.
	1 week	151	162	168	144	126	119
	2 weeks	95	98	92	94	95	90
	3 weeks	63	65	76	72	69	77

important factor determining the later growth response when the diet was fed *ad libitum*. Table 4 gives a comparison of the rate of growth of individuals maintained singly, and populations, both reared in constant temperature environments. The growth of the individual kept at 32.2°C. is very similar to the average number of a population kept at 18.3°C.

Table 5 shows the voluntary food intake of the individual is less than that of the average intake of the population. It would appear that by their general activity, members of a population stimulate each other to eat. There was a very noticeable difference in the activity of populations reared at 23.9° or 21.1°C. compared with those reared at 12.8°C.,

those at 23.9°C. being much more vigorous. However, at 12.8°C., the average intake of a population is greater than that of the individual at 32.2°C. The efficiency of food utilisation (Table 5) is best at 32.2°C. for the chicken maintained singly. Kleiber and Dougherty (1934) show that for chickens of up to 15 days of age, the efficiency of energy utilisation is maximal at 35.0° to 37.8°C. In the same table of results, they also show that growth rate is maximal at 21.1°C. In this problem, a distinction must be drawn between efficiency of food use

TABLE 5

*Food intake and the efficiency of food utilisation during the first fortnight after hatching for birds maintained singly, or in populations, at diverse temperature*

		Brooding temperatures				
		32.3°C.	23.9°C.	15.6°C.	7.2°C.	
A. Singly reared						
Food intake		201	250	272	208	
Food utilisation efficiency		0.67	0.46	0.34	0.20	
B. Populations	26.7°C.	23.9°C.	21.1°C.	18.3°C.	15.6°C.	12.8°C.
Food intake	252	289	249	224	221	219
Food utilisation efficiency	0.62	0.60	0.61	0.62	0.60	0.56

and the growth rate. A comparison of the response of the individual and the population in Table 5 suggests that the individual responds to a temperature stimulus by increasing its food intake whereas the population does not. If the comparison of individual intake had been made on a unit body weight basis, the chickens at 7.2°C. consume three times the food of those kept at 32.2°C. The efficiency of food utilisation of the populations is very similar from 26.7° to 12.8°C. whereas the individual efficiencies decrease threefold. Because the food utilisation efficiency for the populations is similar, ambient temperature did not, therefore, significantly alter the metabolic heat loss. The population responds to the temperature stimulus, not by increasing food intake, but by a behavioural reaction whereby they huddle more closely. The differences in the efficiencies of food utilisation of the individual can be explained by the increasing metabolic heat losses at the cooler temperatures. The percentage of the energy intake used for heat production by these individually maintained chickens during the first fortnight after hatching was 37.7 per cent at 32.2°C., 43.7 per cent at 23.9°C., 48.8 per cent at 15.6°C. and 56.5 per cent at 7.2°C.

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ENVIRONMENTAL TEMPERATURE AND  
EGG PRODUCTION

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THREE EXPERIMENTS have been carried out with laying hens housed under defined temperature regimes within the 18-30°C. range.

In the first two experiments where various temperature environments were compared, more efficient production was obtained when the environmental temperatures approximated to 30°C. as compared with 20°C. In the third experiment where two diets containing different energy levels were fed to layers housed at 30°C., a diet containing 42.5 per cent of barley was associated with more efficient egg production, than a similar diet of higher energy level where the barley was replaced by wheat.

Reasons for the apparent contradictions between these results and other data from hens housed at 30°C. reported in the literature are discussed.

*Introduction*

environment around 15°C. was superior to a non-controlled temperature environment where the daily minimum temperatures fluctuated between 5°C. and 10°C. for long periods.

Voluntary food intake decreases as environmental temperature is raised, and if the two extremes of temperature in Table 1 are compared, then a difference in food intake of approximately 40 per cent is noted.

TABLE 1

*Egg production as influenced by environmental temperature  
(data adapted from Ota, 1960)*

Air temperature °C.	Egg number per 100 hens per day	Egg size g.	Daily feed intake g./ bird	Food conversion kg./dozen eggs
-5	26	57.5	186	8.63
3	65	56.7	158	2.95
8	74	56.4	150	2.32
13	78	55.8	141	2.18
18	75	55.0	132	2.09
24	68	53.8	122	2.18
29	56	52.4	113	2.32

TABLE 2

*Egg production as influenced by environmental temperature  
(data adapted from Mueller (1961))*

Air temperature	Egg production per survivor in 285 days	Food intake g./bird/day	Food conversion kg./dozen eggs	Egg size g.
Constant 13°C.	179 (62.9%)	128.0	2.46	58.9
Constant 32°C.	140 (49.1%)	86.5	2.36	50.4
13-32-13°C. cycling with a gradually changing tempera- ture throughout the 24 hr.	190 (66.7%)	109.2	2.00	56.7

TABLE 3

*The effects of constant versus fluctuating temperature environments on the performance of laying pullets (White Leghorns fed a diet containing 2,640 (M.E.) kcal./kg.)*

House temperature regime	Fairly constant. Minimum temperatures higher than 13°C.	Fluctuating. Minimum temperatures often 5°C. and below
Forty weeks egg production, per cent	72.7	70.7
Food consumption, g./bird/day	125	130
Food consumption, kcal. (M.E.)/bird/day	329	341
Food conversion, kg. food/dozen eggs	2.06	2.20
Egg weight, g.	60.2	60.8

(Data of Peterson, Sauter, Conrad and Lampman, 1960.)

This variation in voluntary food intake with environmental temperature, is an energy-regulating mechanism; the requirements for other nutrients remains fairly static. Thus, the National Research Council (1960) states that protein and amino acid requirements of laying hens remain as fairly constant absolute quantities irrespective of the level of food intake. However, requirements are usually expressed as percentages of the diet rather than as absolute amounts, and usually levels of essential nutrients are not adjusted according to feed intake. Thus, at high food intakes surplus quantities of these essential nutrients may be consumed, whereas at low food intakes nutritional deficiencies may

TABLE 4

*Calculations on the daily intake of methionine and tryptophan consumed in Mueller's (1961) experiments (shown in Table 2)*

	Methionine	Tryptophan
National Research Council (1960) stated requirements expressed as a percentage of diet	0.28%	0.15%
National Research Council (1960) requirements expressed as g./bird/day, assuming that the N.R.C. figures refer to a consumption of 114 g./bird/day	0.32 g.	0.17 g.
Calculated intake (g./bird/day) at 13°C. constant	0.34 g.	0.19 g.
Calculated intake (g./bird/day) 13°-32°-13°C. cycling	0.31 g.	0.17 g.
Calculated intake (g./bird/day) at 32°C. constant	0.25 g.	0.13 g.

occur. If the data of Mueller (1961) given in Table 2 are reconsidered, it can be demonstrated, as shown in Table 4, that the poor production of the hens housed in the 32°C. constant environment may have been due to deficiencies of methionine and tryptophan.

This hypothesis is supported by the data of Bray and Gessell (1961), who housed hens under constant temperature environments of between 6°C. and 30°C. for short-term collection periods. In these experiments it was shown that provided the daily food intake contained 15 g. of crude protein, with 0.49 g. of the sulphur containing amino acids, egg production was similar at all environmental temperatures even though food intake varied between 120 g./bird/day at 6°C. and 90 g./bird/day at 32°C.

obtained in one experiment, but were obtained from similar stock housed as control birds for different observations in the ammonia experiments. Because of their low food consumption and good egg production, the food utilisation efficiency in the 29°C. environment was superior to any results we had previously obtained. In this series of observations as well as in subsequent experiments, hens housed at 29°-30°C. showed no symptoms of heat stress.

TABLE 5

*Diets used in the environmental temperature experiments*

	EL 21	EL 24	EL 30	EL 31
Yellow maize meal	45	22	25	25
Ground barley	—	—	42.5	—
Ground wheat	—	27	—	42.5
Ground milo	20	27	—	—
White fishmeal	—	5	7.5	7.5
Soyabean meal	20	7.5	10	10
Pinhead limestone	5	4	7.5	7.5
Layers supplement*	5	5	5	5
Fat†	5	2.5	2.5	2.5
Calculated analyses				
Metabolisable energy (kcal./kg.)	3075	3150	2740	2950
Calcium (per cent)	3.2	3.2	4.6	4.6
Crude protein (per cent)	15.6	15.0	16.1	17.0
Methionine (per cent)	0.35	0.36	0.42	0.44

\* Described by Smith and Lewis (1964); containing 0.10 per cent added DL-methionine.

† H.E.F., No. 1, supplied by Proctor and Gamble Ltd., Newcastle-upon-Tyne.

TABLE 6

*Observations on the performance of White Leghorn pullets housed at two environmental temperatures. The figures refer to 54 pullets/treatment, fed diet EL 21 (Data of Charles, Payne and Lamming, 1963)*

	18°C.	29°C.
Environmental temperature		
Relative humidity	67 per cent	51 per cent
Egg production (per cent hen week)	78.7	79.6
Food consumption (g./bird/day)	119.0	95.6
Shell thickness (mm.)	0.338	0.333
Egg weight (g.)	61.4	58.3
Food conversion (kg./dozen eggs)	1.83	1.47

The second experiment was of a pilot nature, involving 12 rooms; each room contained 12 hens. There were six environmental temperature treatments; thus each temperature treatment was performed using only 24 hens housed in 8 triple-bird cages. The results of this experiment are shown in Table 7, and as in the previous experiment, the efficient performance of the stock housed at high temperatures was reiterated. There was some indication that the performance under fluctuating temperature environments was superior to constant ones, especially if average temperatures are compared.

TABLE 7  
*Summary of results obtained from the second environmental experiment at the University of Nottingham (Lincoln, 1964)*  
*The results refer to White Leghorn pullets, housed under continuous lighting, for 16 weeks. Fed diet EL 24*

Environment	Least significant difference P < 0.05	Temperature							
		18°C. constant	24°C. constant	30°C. constant	30°C. for 15 hr. 18°C. for 9 hr.	30°C. for 9 hr. 18°C. for 15 hr.	24°C. for 9 hr. 18°C. for 15 hr.	20.3°C.	
Average temperature		18°C.	24°C.	30°C.	25.5°C.	22.5°C.			
Egg production per cent hen day	2.17	78.1	81.4	84.2	84.4	82.6	81.9		
Food consumption g./bird/day	2.43	114.7	108.9	105.3	107.9	111.8	115.9		
Egg size (g.) after 16 weeks	0.45	57.3	55.9	55.8	55.6	56.0	56.3		
Food conversion (kg./doz.)	0.07	1.76	1.60	1.50	1.53	1.62	1.70		
Food conversion (g. food/g. eggs)	0.10	2.71	2.69	2.45	2.49	2.69	2.77		



In the second experiment all the stock gained more than 3 g. live-weight per day; which seemed to be an unusually large amount, thus, in a subsequent experiment the prolonged performance of White Leghorn hens housed at 30°C. is being studied, when fed diets containing two different energy levels (diets EL 30 and EL 31 given in Table 5). The results of this experiment for the period of 260 days from first egg are shown in Table 8. Excellent production has been obtained from the

TABLE 8

*Results of the third environmental experiment—72 White Leghorn pullets per treatment housed in triple bird cages at 30°C. constant for the period 0-260 days from the first egg*

	Medium energy diet EL 30	Higher energy diet EL 31
<i>0-260 day period</i>		
Number of eggs/hen (hen housed)	197.5	180.0
Food consumption (g./bird/day)	99.2	96.6
M.E. intake (Cal./bird/day)	270	285
Liveweight gain (g./bird/day)	0.86	1.32
Mean egg size (g.)	57.6	57.4
Food conversion (kg./dozen eggs)	1.55	1.67
Food conversion (g. food/g. egg)	2.24	2.43
<i>231-260 day period</i>		
Per cent lay (hen/day)	75.2	77.8
Food intake (g./bird/day)	93.5	86.7
M.E. intake (Cal./bird/day)	256	255

hens fed either diet; the slightly lower production on the higher energy ration has been due to fluctuations rather than a prolonged falling off in egg production. These fluctuations may have been due to over-consumption of energy, or to inadequacies within the protein component of the diet.

### Conclusions

1. Experiments reported in the literature indicate that 10-15°C. is the ideal environmental temperature for laying hens. However, the decreased production at higher temperatures may have been due to nutritional inadequacies mediated by low food consumption.

2. On feeding diets containing high levels of methionine and other essential nutrients, this reduction in performance at high environmental temperatures has been largely overcome, and feed efficiency has been improved. However, egg size has still deteriorated as environmental temperatures have been raised.

3. There is a need for considerable experimentation on the optimal temperature requirements of laying stock. Such experiments must be carried out using diets and lighting patterns which are carefully defined.

4. In the interpretation and adaptation of the results of any

temperature experiment for commercial application, care must be taken in the design of the housing used, especially regarding insulation, ventilation and heating standards.

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# CONTROLLED ENVIRONMENT HOUSING: THE PRACTICAL PROBLEM OF OBTAINING THE PHYSIOLOGICAL OPTIMUM

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## *Synopsis*

THE CONVENTIONAL arrangement for ventilation of poultry houses is to extract the stale air at the ridge and bring the fresh air in at the sides. With the modern wider span house this system can lead to poor distribution of the air and uneven floor air velocities. Methods are described in which the incoming fresh air can be diffused and its velocity so lowered that uniform air movement is ensured across the building. Fans are placed in the wall to remove the adverse effects of wind pressure. Artificial heating can be provided either at the entrance to the duct or as the air passes out of the duct into the house.

## *Introduction*

In recent years the climatic conditions required by poultry have been defined with some accuracy. Our knowledge has been reviewed in detail by Hutchinson (1954), Wilson (1957), Payne (1962), Osbaldiston and Sainsbury (1963) and Harvey (1963). The problem, however, in the poultry industry has increasingly resolved itself into the difficulty of obtaining those conditions defined by the physiologist as being optimal, by economic and practical means.

The aim of this paper is to present some methods whereby this problem may be reduced or relieved, with particular emphasis on air movement and ventilation.

The most important climatic needs of poultry are a correct and reasonably uniform ambient temperature together with a ventilation rate which is principally related to the liberation from the atmosphere of the respiratory moisture of the birds and evaporations from their excretions. The optimum temperature for the day-old chick is in the order of 32° and this reduces to about 13°C. by maturity. It is therefore clear that in temperate climates such as the European, the temperature inside the building will for most of the year have to be kept above the outside temperatures. The required ambient temperature in

the building may be built up in three ways—by artificial heating of the air where appropriate, by retention of the body heat of the birds and by good thermal insulation of the surfaces to retard the heat loss.

Artificial heating is apparently only necessary in the first few weeks of life; later the correct temperature can be generally obtained by the two last mentioned methods. Nowadays with the high density of stocking normally practised, the heat output per square metre of surface area is high and this materially assists in the maintenance of the correct ambient temperature.

In practice the essential problem is to maintain the correct temperature throughout the building and yet obtain the required ventilation rate and an even and draught free movement of air at the position of the birds.

### *Methods*

In the traditional poultry house of narrow span (6 metres to 9 metres) the stale air is normally extracted at the ridge of the house and the fresh air is brought in at the walls through hopper or other forms of inlet openings (see Fig. 1). Modern practice, however, favours a wider

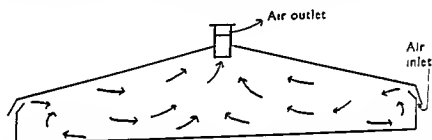


FIG. 1. Diagrammatic cross section of conventional poultry house showing the pattern of air movement indicated by the arrows.

span of house and spans varying from 13.7 metres to 21.3 metres are common. With the conventional arrangement there is a pronounced tendency for considerable variations to exist between the temperature of the incoming fresh air at the side of the house and the stale air at the centre of the building. If the birds are running on the floor the results of this can be a poor distribution of the stock leading to overcrowding in some areas. It is well known that this can have harmful effects on productivity and the incidence of disease.

One method that has been found successful in reducing this unfavourable effect is to diffuse the air as it enters by means of a filter of glass fibre or hessian. On other occasions the diffusion is done successfully by using perforated hardboard. In both cases the air not only enters over a large area at low velocity but the effects of the wind velocity are reduced to a minimum by the buffering ability of the filter or hardboard. There are also available medicated filters which are designed to have the combined effect of diffusing the air and purifying it from micro-organisms that reach the house by windborne spread.

With the method described above there are still considerable

practical difficulties. The walls of the modern poultry house are often low so that it is still difficult to avoid rather steep temperature gradients at floor level. Also, the extracting fans in the ridge are affected by high wind pressures. It can therefore be reasoned that if the air is brought in at the centre of the house and the extracting fans are placed in the walls to move the air in the opposite direction, the distribution of air could be better and the harmful effects of wind pressure on the house could be nullified by suitable mounting of the fans.

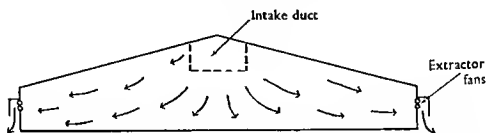


FIG. 2. Diagrammatic cross section of poultry house with side extraction and diffused air intake showing direction of air movement.

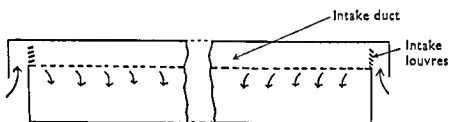


FIG. 3. Diagrammatic longitudinal section of poultry house of the same type as in Fig. 2.

A practical system that has been developed following these last-mentioned principles functions as follows:

- (i) Multiple extracting fans are placed in the side walls with external baffles to prevent wind pressure effects (see Fig. 2).
- (ii) A large central duct is placed under the ridge of the building and is designed to be of such an area that the air velocity along its length is not in excess of 305 m./min. (see Fig. 3).
- (iii) The duct is opened at each end to the atmosphere; controllable louvres allow sufficient fresh air entry but protect the duct from rain or snow.
- (iv) The form of perforated hardboard used has 2,800 holes per square metre, each aperture being 0.51 cm. in diameter. Using this arrangement in a 21.3 metre wide house with an intake duct of 4.65 square metres area it has been found that the air velocity as little as 0.305 metres away from the duct is under 12.2 metres per minute during the minimum winter ventilation. This is well within the critical requirements of the young chick (Payne, 1961). Smoke tests carried out with this system show a uniform and even movement of fresh air from the duct to the extracting fans.

- (v) Heating may be applied to the incoming air either in or outside the duct. The most usual arrangement is to heat the air at one or both ends of the duct. An alternative arrangement that may be used is to place radiant or other heaters within the house but close to the duct so they heat the fresh air as it emerges. With both arrangements the need for additional brooding heat is eliminated.

### Conclusions

Initial experience with this system of central diffused air entry and wall extraction confirms that it can produce an even distribution of birds on the floor and an absence of measurable temperature or air velocity variation across the house. Well-designed baffles over the extracting fans have also prevented the wind pressure exerting an unfavourable effect on their mechanical efficiency. The arrangement has proved suitable for all ages of birds kept on the floor and the quantities of ventilation and of heat can be simply regulated at one control point.

Variations of this method have also been used for laying birds in batteries and for chicks in tier-brooders; the benefits of air diffusion are probably even more important with birds kept in cages because they have no means of movement to avoid cold-air draughts.

### Acknowledgement

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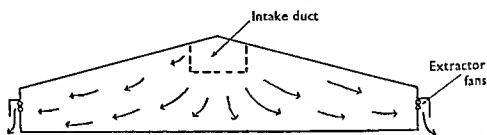


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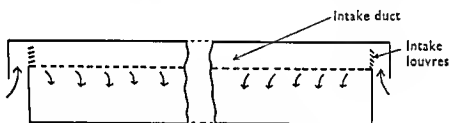


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PART V

PHARMACOLOGY  
AND GENERAL PHYSIOLOGY

## ANTICHOLINESTERASE DRUGS IN THE FOWL

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*Synopsis*

EVIDENCE is reviewed which indicates that the cholinesterase enzymes of the domestic fowl differ from those of mammals chiefly in their inability to combine with bisquaternary substrates and inhibitors. On the basis of this difference it is suggested that it may be possible to synthesise insecticides of the organo-phosphorus type which would be relatively harmless to poultry and possibly to other birds.

*Anticholinesterase Drugs*

Pharmacologists have long made use of certain avian nerve-muscle preparations when studying the mechanism of action of neuromuscular blocking drugs. One such preparation is the sciatic nerve-gastrocnemius muscle of the anaesthetised domestic fowl (Brown and Harvey, 1938*a, b*; Zaimis, 1959; Bowman, Callingham and Goldberg, 1961). Ginsborg (1960) has shown that a large proportion of the muscle fibres of this muscle are multiply innervated so that the membranes of the individual muscle fibres contain a large number of motor end-plates. Such muscle fibres are therefore sensitive to chemical agents like acetylcholine over a large amount of their surface area. As a result these muscles respond to injected depolarising drugs by a quick contraction which is followed by a sustained non-propagated contracture. Fig. 1 illustrates an oscilloscope recording of the isometric tension and the electromyogram of the fowl's gastrocnemius muscle. An intravenous injection of the depolarising drug carbolonium (imbretil) caused a burst of propagated action potentials from the muscle accompanied by

a quick contraction. Propagated action potentials then disappeared but a sustained contracture of the muscle persisted. This characteristic contractural response of the fowl's gastrocnemius muscle to depolarising drugs enables this type of drug to be readily distinguished. The response does not occur in focally innervated muscles (innervated by only one branch of a nerve fibre) which include the majority of mammalian muscles.

Fig. 2 illustrates the use of the fowl gastrocnemius muscle preparation when studying the mechanism of action of neuromuscular blocking drugs. Clinically useful neuromuscular blocking drugs may be divided

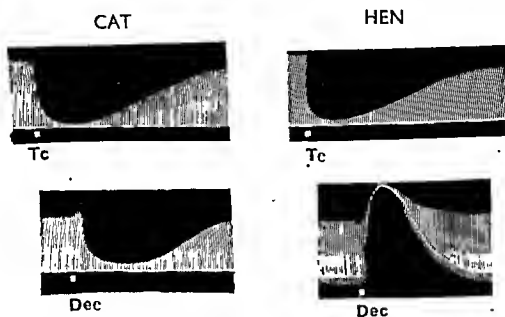
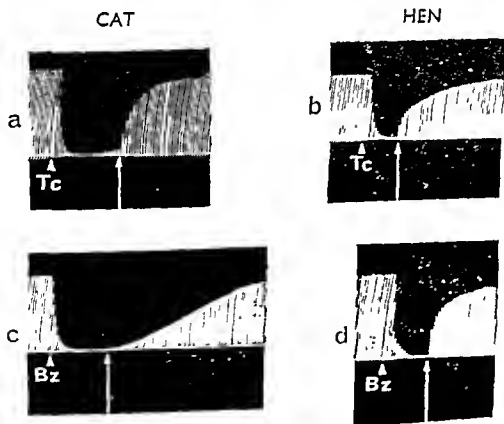


FIG. 2. Cats and hens under intravenous chloralose anaesthesia. Maximal twitches of cat tibialis anterior muscles (left-hand panels) and hen gastrocnemius muscles (right-hand panels) elicited once every 10 sec. by stimulation of the motor nerves. At Tc, tubocurarine was injected intravenously; 0.4 mg./kg. in the cat and 1 mg./kg. in the hen. At Dec, decamethonium was injected intravenously; 40  $\mu$ g./kg. in the cat and 10  $\mu$ g./kg. in the hen.

into two main groups—those, such as tubocurarine, which block the access of acetylcholine to its receptors, and those such as decamethonium which are depolarising drugs with an action resembling that of acetylcholine itself, but which are stable to cholinesterase. Both types produce a flaccid paralysis in the muscles of man and other mammals. The left-hand panels of Fig. 2 illustrate twitches of a cat limb muscle in response to motor nerve stimulation. Both tubocurarine and decamethonium caused neuromuscular block and, although their mechanisms of action are entirely different, there is not a great deal of difference between the pictures produced in the mammal. However, in the muscle of the fowl the difference is immediately obvious. Tubocurarine, and drugs acting like it, produce a similar picture to that obtained in the mammal (upper right-hand panel of Fig. 2), but decamethonium and other depolarising drugs produce the characteristic spastic paralysis

already described (lower right-hand panel of Fig. 2). The avian muscle therefore provides a quick method for distinguishing between these two classes of blocking drugs.

It was while studying the mechanism of action of benzoquinonium in this way (Bowman, 1958) that the first clue was obtained that the cholinesterase enzymes in the fowl differ from those in the mammal. Benzoquinonium is a neuromuscular blocking drug which, for a brief period, was used as an adjuvant to surgical anaesthesia in man. In the cat benzoquinonium produced a paralysis resembling that produced by tubocurarine, but it differed from tubocurarine in one important respect which is illustrated in Fig. 3. The block produced by tubocurarine was readily antagonised by anticholinesterases, such as neostigmine (Fig. 3a) but that produced by benzoquinonium was not (Fig. 3c). This was also so in man and was the main reason for discontinuing its use. The recovery shown in Fig. 3c was simply the normal rate of recovery; it was not hastened by neostigmine. Lack of antagonism by anticholinesterase drugs is a characteristic of the depolarising type of blocking drug and it was therefore decided to study the effect of benzoquinonium in the fowl (Bowman, 1958). Benzoquinonium was



much more potent in the fowl than in the cat and there was no evidence of any depolarising activity. In fact, the block resembled that produced by tubocurarine and, surprisingly, it was well antagonised by neostigmine (Fig. 3*a*) unlike its action in the cat. In addition to its neuromuscular blocking action, benzoquinonium was found in the cat and other mammals to possess considerable anticholinesterase activity (Hoppe, 1951; Blaber and Bowman, 1962*a*). Its curare-like action is therefore sufficiently pronounced for it to produce block in spite of its own anticholinesterase activity. The anticholinesterase activity of benzoquinonium provides an explanation of the inability of neostigmine to antagonise its neuromuscular blocking activity, since if the cholinesterase is already inhibited by benzoquinonium itself, neostigmine would not be expected to exert any further effect. The fact that benzoquinonium was a more powerful blocking agent in the fowl than in the cat, and that its action was readily antagonised by neostigmine suggested that benzoquinonium itself was without anticholinesterase activity in the fowl.

In a subsequent study these observations with benzoquinonium

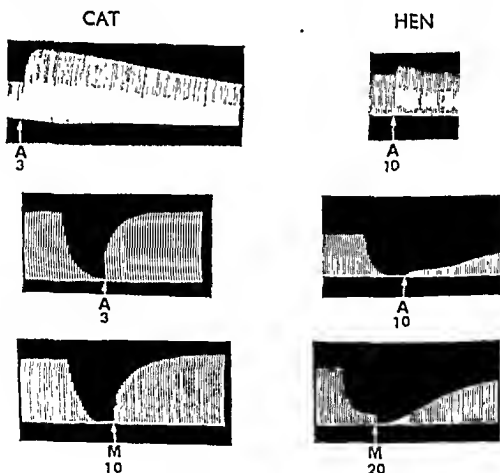


FIG. 4. Cats and hens under intravenous chloralose anaesthesia. Details as in Fig. 2. At A, ambenonium and at M, methoxyambenonium injected directly into the arterial supply to the muscle; the numbers denote the doses in  $\mu\text{g}$ . In the lower four panels, neuromuscular transmission was blocked with intravenous tubocurarine (0.4 mg./kg. in the cats and 1 mg./kg. in the hens) before ambenonium or methoxyambenonium were injected.

were extended and the effects of other anticholinesterase drugs on the muscles of the cat and of the fowl were also examined (Blaber and Bowman, 1962*b*). Two of the anticholinesterase drugs chosen for study were ambenonium and methoxyambenonium. Like benzoquinonium, these two drugs contain 2 quaternary nitrogen atoms (Fig. 7).

Fig. 4 (left-hand panels) illustrates that in the cat, ambenonium was highly active in potentiating muscle twitches elicited by motor nerve stimulation and in antagonising tubocurarine. Methoxyambenonium was also a powerful anti-curare agent. These effects are characteristic

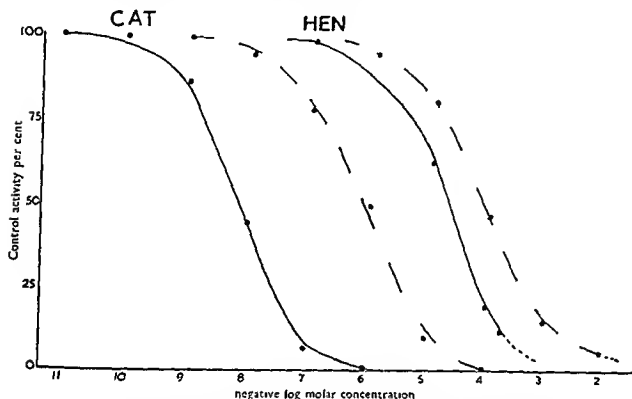


FIG. 5. Inhibition of muscle acetylcholinesterase from the cat and the hen by ambenonium (continuous curves) and methoxyambenonium (broken curves). Acetylcholine chloride (0.0138 M) was used as substrate.

of anticholinesterase drugs. In the fowl, on the other hand (Fig. 4, right-hand panels) the effects of ambenonium were very weak, and methoxyambenonium was completely without anti-curare action. In fact, it slightly increased the tubocurarine block. The weak effects of ambenonium in the fowl illustrated in Fig. 4 (right-hand panels) were the maximal obtained in any experiment and any increase in the dose actually had the opposite effect and the contractions were depressed.

Manometric studies with the Warburg apparatus were then carried out to determine the inhibiting activity of ambenonium and methoxyambenonium against the acetylcholinesterase in homogenates of cat and hen skeletal muscle. Fig. 5 illustrates the results obtained. The concentration of ambenonium necessary to cause 50 per cent inhibition of the cat muscle enzyme was about  $10^{-8}$  M. But the concentration necessary to cause the same amount of inhibition of the fowl muscle enzyme was much greater, around  $10^{-4.5}$  M. That is, ambenonium

was over 3,000 times less potent against the enzyme from the hen than against that from the cat, and these *in vitro* results therefore supported those observed *in vivo*.

In similar experiments with anticholinesterase drugs containing only one charged nitrogen atom, we found little difference between the cat and the fowl enzymes. These anticholinesterase drugs included neostigmine, physostigmine, edrophonium and pyridostigmine as well as several others. Fig. 6 illustrates experiments with neostigmine which serve to illustrate this point. Neostigmine potentiated the twitch

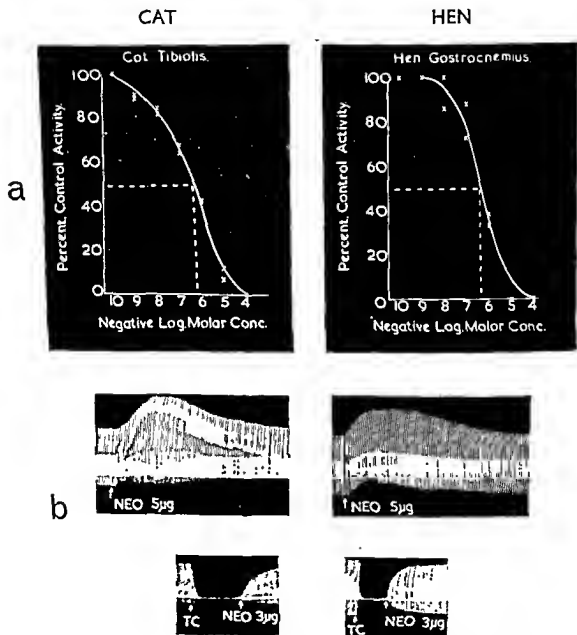


FIG. 6. (a) Inhibition of muscle acetylcholinesterase from the cat and the hen by neostigmine. Acetylcholine chloride (0.0138 M) was used as substrate.

(b) Cats and hens under intravenous chloralose anaesthesia. Details as in Fig. 2. At *Neo*, neostigmine was injected directly into the arterial supply to the muscles; the numerals denote the doses of neostigmine in  $\mu$ g. In the lower panels, neuromuscular transmission was first blocked by tubocurarine (0.3 mg./kg. in the cat and 0.8 mg./kg. in the hen).

and antagonised tubocurarine in similar doses in both species (Fig. 6b). The curves plotted from manometric studies were similar. Fifty per cent inhibition of both enzymes being produced by concentrations of the order of  $10^{-6.5}$  M. (Fig. 6a) Table 1 gives the relative anticholinesterase activities of some of the compounds compared with neostigmine as standard. Thus ambenonium was about 36 times more potent than neostigmine in the cat but 200 times less potent than neostigmine in the hen. Methoxyambenonium and benzoquinonium were about  $\frac{1}{5}$  as potent as neostigmine in the cat but only  $\frac{1}{500}$  to  $\frac{1}{1000}$  as potent in the hen.

TABLE 1

*Relative anticholinesterase activities of some bisquaternary compounds compared with neostigmine, arbitrarily given the value 100. Results obtained using cat and hen skeletal muscle as the source of acetylcholinesterase, and acetylcholine chloride (0.0138 M) as substrate (from Blaber and Bowman, 1962b)*

	Cat	Fowl
Neostigmine	100	100
Ambenonium	3,654	0.5
Methoxyambenonium	22.3	0.1
Benzoquinonium	21.6	0.2

It therefore seemed that the hen muscle enzyme differed from that in the mammal chiefly in that it could not combine with compounds possessing 2 quaternary nitrogen atoms, and this brought to mind an effect frequently observed during student demonstrations. Namely that the contractural response of the fowl muscle to the depolarising drug succinylcholine is as long-lasting as that produced by decamethonium (Blaber and Bowman, 1962b). In man and in the cat, succinylcholine produces a very short-lasting neuromuscular block compared to decamethonium because it is quickly hydrolysed by the pseudocholinesterase of the plasma. Decamethonium is not inactivated in this way. The obvious explanation is that the bisquaternary substrate, succinylcholine, like the bisquaternary inhibitors, cannot combine with the enzyme in the fowl and this was confirmed in manometric studies with cat and fowl plasma using succinylcholine as substrate. In similar experiments there was seen to be little difference between the abilities of the two types of plasma to hydrolyse the monoquaternary substrate acetylcholine, and these results therefore further supported the idea that both the true and the pseudocholinesterases of the fowl combine to only a very small extent with bisquaternary compounds.

Subsequently, Blaber and Cuthbert (1962) made a detailed manometric study of the reaction of a wide variety of substrates and inhibitors with the cholinesterases from brain, gut, plasma and skeletal muscle of the fowl. They observed a number of minor differences between the mammalian and avian enzymes. The pseudocholinesterase of the fowl was a propionylcholinesterase with properties intermediate



between the true and pseudo-enzymes of the mammal. The true cholinesterase of the fowl was an acetylcholinesterase with properties on the whole similar to its mammalian counterpart. Blaber and Cuthbert confirmed, for all tissues, the prime difference concerning the inactivity of bisquaternaries and added another to the list. This was the compound 62C47 (Fig. 7), a specific inhibitor of the

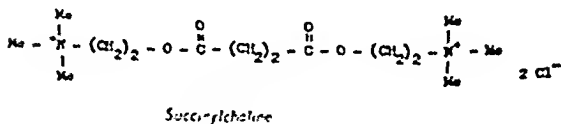
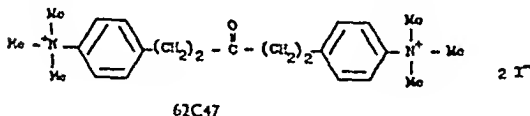
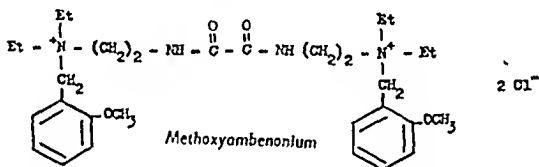
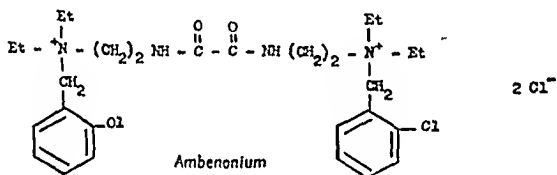
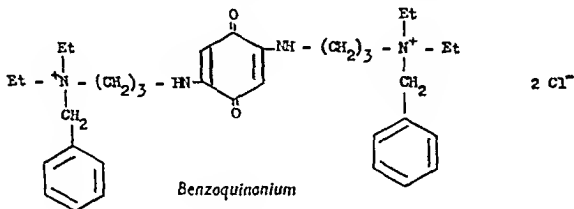


FIG. 7. Formulae of bisquaternary compounds studied.

acetylcholinesterase present in rat brain (Fulton and Mogey, 1954), but found to be almost inactive in the chicken.

The fact that the bisquaternary compounds were inactive in the fowl while the potencies of those containing only one nitrogen were found to be of the same order in both species suggests that there may be differences in the position of the active sites on the two types of enzyme. Analysis (for references see Cohen and Oosterbaan, 1963) of the molecular forces acting on acetylcholinesterase carried out with a large number of substrates and inhibitors, has shown that the active surface of the enzyme has two functionally and spatially separated subsites—an anionic and an esteratic site (Fig. 8). The anionic site attracts the cationic head of the substrate by coulombic and van der Waal's forces.

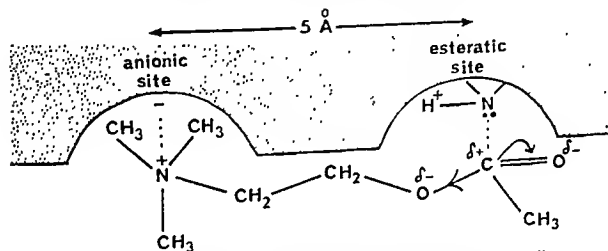


FIG. 8. Diagram of the initial interaction between acetylcholine and acetylcholinesterase.

The esteratic site carries out the hydrolysis and has both an acidic and a basic or nucleophilic group, the latter forming a covalent bond with the electrophilic carbon of the carbonyl group of the substrate. It has also been shown that on the single cholinesterase molecule there may be as many as 48 different receptors for acetylcholine (Michel and Krop, 1951). In the bisquaternary compounds the two quaternary nitrogens are separated by a distance of about 12 Å, which is approximately twice the distance between the quaternary nitrogen and the ester group in acetylcholine. This suggests that 2 sets of receptors on the enzyme molecule may be involved in the reaction with a bisquaternary and Fig. 9 illustrates a possible difference in the arrangements of the active sites on the two types of enzyme which would account for the observed effects. In the mammal, two negatively charged anionic sites may attract and hold the positively charged nitrogens as illustrated in the upper diagram of Fig. 9 so that the esteratic site is masked and prevented from functioning. In the enzyme from the fowl, an arrangement like that in the lower half of Fig. 9 would then explain the inactivity of the bisquaternaries, although clearly both types of enzyme would combine with monoquaternaries such as neostigmine and acetylcholine. The diagram is probably

oversimplified and may be only one of several possible arrangements. Nevertheless, it does give an explanation which fits the facts and serves as a model, the accuracy of which might be tested by synthesising and studying a variety of bisquaternaries of all types.

The possible importance of the fact that there is a difference in the two types of enzyme may be as follows. The commonly used insecticides are anticholinesterases of the irreversible organo-phosphorus type and these are highly toxic not only to insect life but also to other forms

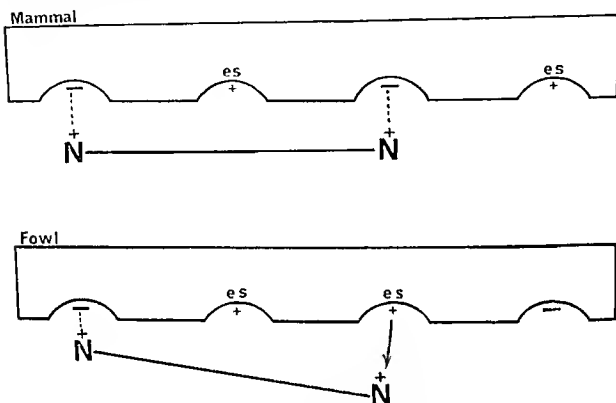


FIG. 9. Diagram illustrating a possible difference between the arrangement of the active sites on mammalian and fowl cholinesterase.

which they kill because of their ability both to cause an acute increase in the levels of acetylcholine in the central nervous system and to cause a chronic demyelination of nerve fibres. Whatever the lethal effect in insects is due to, the fact remains that high insecticide activity is associated with potent anticholinesterase activity in the mammal. Since there is this difference in the enzymes it might well be possible for organic chemists to synthesise a molecule, not necessarily a bisquaternary, which would be a potent anticholinesterase in the mammal and a powerful insecticide but relatively harmless to poultry. Furthermore, if the characteristics of the fowl cholinesterase turn out to be true for other avian species, such a compound should also be relatively harmless to wild bird life and may do something to quell some of the criticism of the use of this type of insecticide which is prevalent at the moment. At least one bisquaternary organo-phosphorus compound has been synthesised (Fig. 10). It is a powerful anticholinesterase in the mammal (Hazaard, Cheymol, Chabrier and Carayon-Gentil, 1956)

but its potency in the fowl and as an insecticide is not known. Insects are known to be resistant to the lethal effects of quaternary compounds, possibly because the insect nervous system is protected by a lipoidal

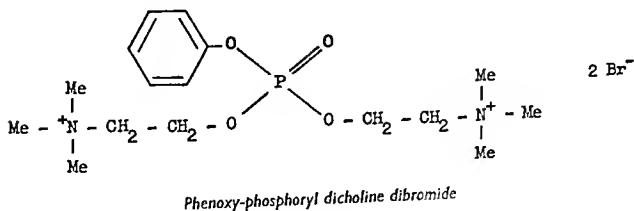


FIG. 10. A bisquaternary organo-phosphorus anticholinesterase.

barrier which retards the penetration of ionised compounds. It may therefore be that a compound containing two tertiary nitrogens at the optimal distance apart would exhibit more selective toxicity.

### *Acknowledgements*

I am grateful to the British Egg Marketing Board for financial support during the more recent of the experiments described.

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# PHARMACOLOGICAL RESPONSES OF THE ISOLATED OESOPHAGUS AND CROP OF THE CHICK

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## *Synopsis*

RESPONSES OF isolated preparations of the oesophagus and crop of chicks to parasympathetic nerve stimulation and to various drugs are described. These tissues were found to be essentially similar to mammalian smooth muscles in their reaction to pharmacological agents, and they provide useful isolated preparations for studying the action of drugs and for student practical classes. A sensitive preparation of the crop for the assay of 5-hydroxytryptamine is described. Owing to seasonal variations in sensitivity, this preparation is likely to be of use only during the summer months.

## *Introduction*

This paper describes the effects of various drugs on isolated parasympathetically innervated preparations of the oesophagus and crop of chicks, and the use of a preparation of the chick crop for the biological assay of 5-hydroxytryptamine. These preparations have been briefly described elsewhere (Bowman and Everett, 1964; Everett, 1964).

## *Materials and methods*

Male chicks (Silver Link) aged from 1 to 6 days after hatching were starved overnight and then killed with ether.

## *Oesophagus*

The method was identical with that previously described (Bowman and Everett, 1964). The oesophagus above the crop was removed together with the right vagus nerve trunk and the adhering jugular vein, and suspended in Krebs's solution continuously gassed with 5 per cent carbon dioxide in oxygen at 32°C. The central end of the nerve was passed through submerged platinum stimulating electrodes of the type described by Burn and Rand (1960). Contractions of the oesophagus

were elicited by stimulation of the nerve with rectangular shocks of 0.5 msec. duration. The strength of the shocks was such that the contractions for a given frequency of stimulation were maximal. The contractions were recorded on smoked paper by means of an isotonic frontal writing lever.

In some experiments stimulation was applied coaxially between an electrode (the anode) in the lumen and a second electrode (the cathode) in the external fluid. This type of stimulation has been shown in guinea-pig ileum to stimulate post-ganglionic cholinergic nerve endings preferentially (Paton, 1955). In the chick oesophagus both pre- and post-ganglionic fibres were excited by this type of stimulation, and the ganglion blocking drug hexamethonium ( $4 \mu\text{g./ml.}$ ) was therefore added to the reservoir of Krebs's solution to restrict the effective stimulation to the post-ganglionic fibres. Stimuli of 0.5 msec. duration and supramaximal strength were used to excite the oesophagus coaxially.

#### *Whole Crop.*

Activity of the crop was recorded by ligating the upper end at its junction with the oesophagus, and tying the opening into the lower oesophagus over a tube connected to a manometer filled with Krebs's solution. The crop was immersed in a 100 ml. bath of Krebs's solution at  $32^{\circ}\text{C.}$  and gassed with 95 per cent  $\text{O}_2$  and 5 per cent  $\text{CO}_2$ . The attached right vagus nerve was stimulated as in the experiments on the oesophagus. Before studying the effects of drugs or of nerve stimulation the manometer was raised sufficiently to expand the crop greatly but not enough to increase the internal pressure. Volume changes in the expanded crop were recorded on smoked paper by means of a floating pointer in the manometer.

#### *Assay of 5-HT*

A transverse strip about 4 mm. wide was cut from each opened crop taking great care not to stretch the tissue. The strip was suspended in a 1 ml. bath of Krebs's solution bubbled with 95 per cent  $\text{O}_2$  and 5 per cent  $\text{CO}_2$  at room temperature ( $19\text{--}22^{\circ}\text{C.}$ ) as previously described (Everett, 1964). Contractions in response to standard and test doses of 5-hydroxytryptamine were recorded on smoked paper.

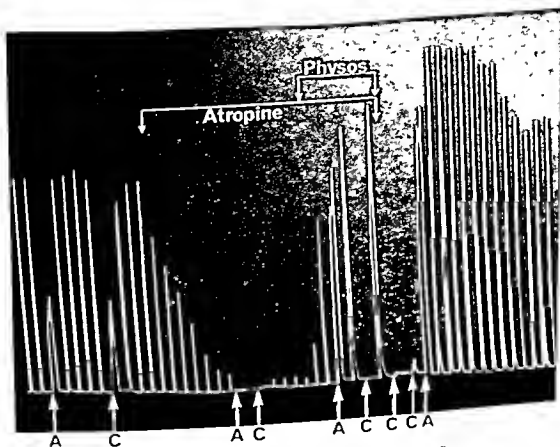
The composition of the Krebs's solution used in these experiments was as follows: g./l.,  $\text{NaCl } 6.95$ ,  $\text{KCl } 0.34$ ,  $\text{CaCl}_2 0.28$ ,  $\text{KH}_2\text{PO}_4 0.162$ ,  $\text{MgSO}_4 0.294$ ,  $\text{NaHCO}_3 2.1$ , dextrose 2.

#### *Experimental results*

Even at the relatively low temperature of  $32^{\circ}\text{C.}$  the oesophagus preparations occasionally exhibited spontaneous pendular movements. As in other species, these movements appeared to be myogenic in

origin and independent of functional nervous tissue. They were unaffected by antagonists of acetylcholine, histamine and 5-hydroxytryptamine, and by anticholinesterase drugs. They were still present on rewarming the tissue to 39°C. after storing it at 2-4°C. for 72 hr. although responses to nerve stimulation were abolished by this treatment. Regular stimulation of the nerve for periods of 10 sec. every 2 min. inhibited and usually completely abolished any spontaneous pendular movements of the oesophagus. On stopping the stimulation, the spontaneous activity usually gradually returned.

The preparation of the oesophagus behaved in a qualitatively similar way to mammalian smooth muscle preparations in its responses to acetylcholine and related drugs, to adrenaline and related drugs and to histamine and 5-hydroxytryptamine. The effects of these substances were blocked by the appropriate antagonists. Records illustrating some of these drug effects have been previously published (Bowman and Everett, 1964). Fig. 1 illustrates responses to nerve stimulation and to acetylcholine, carbachol, atropine and physostigmine. Atropine (0.01  $\mu\text{g./ml.}$ ), added to the reservoir of Krebs's solution, abolished the responses to nerve stimulation and those to acetylcholine and carbachol.



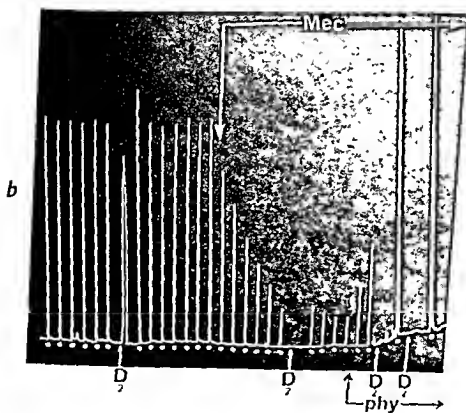
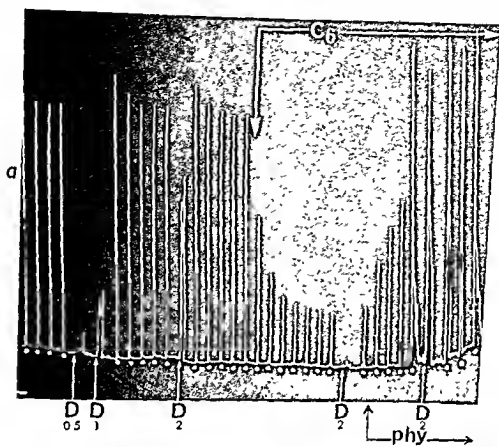


Physostigmine ( $0.2 \mu\text{g./ml.}$ ), added in the continued presence of atropine, restored and potentiated the responses to nerve stimulation and to acetylcholine, but the responses to carbachol (which is stable to cholinesterase) remained blocked even after washing the tissue. Clearly the antagonistic action of physostigmine against atropine cannot be explained on the basis of acetylcholine accumulating in the presence of the anticholinesterase and displacing atropine from receptors, as, carbachol remained blocked. The best explanation appears to be that, by inhibiting cholinesterase, physostigmine markedly increased the activity of acetylcholine in relation to carbachol, thereby allowing it to combine with those receptors which were not blocked by atropine.

Neostigmine ( $0.2 \mu\text{g./ml.}$ ) produced effects similar to those of physostigmine except that it was more active in potentiating responses to nerve stimulation than to applied acetylcholine. This might be because potentiation of nerve stimulation depends on inhibition of true cholinesterase, while potentiation of applied acetylcholine may involve inhibition of pseudocholinesterase. Both physostigmine and neostigmine are known to inhibit both types of enzyme but neostigmine may be more selective for the true cholinesterase. Edrophonium, an anticholinesterase drug which is most active at the neuromuscular junction in striated muscle, was without effect, in concentrations up to  $50 \mu\text{g./ml.}$ , on the responses of the oesophagus to nerve stimulation and to acetylcholine. The bisquaternary anticholinesterase ambenonium was also without effect on the chick oesophagus in concentrations up to  $50 \mu\text{g./ml.}$ , supporting the suggestion (Blaber and Bowman, 1962; Bowman, 1965) that the cholinesterases of this species cannot combine with bisquaternaries.

Similar effects to those described above with acetylcholine, carbachol atropine and the anticholinesterase drugs were obtained when the oesophagus was stimulated coaxially in the presence of hexamethonium.

The oesophagus contracted in response to the ganglion stimulant drugs, nicotine ( $5 \mu\text{g./ml.}$ ), tetramethylammonium ( $5 \mu\text{g./ml.}$ ) and dimethylphenylpiperazine (DMPP,  $2 \mu\text{g./ml.}$ ) and these responses, as well as those to nerve stimulation, were depressed by the ganglion blocking drugs hexamethonium ( $2.4 \mu\text{g./ml.}$ ), mecamylamine ( $1.0 \mu\text{g./ml.}$ ) and pempidine ( $1.0 \mu\text{g./ml.}$ ) (Bowman and Everett, 1964). The anticholinesterase drugs physostigmine and neostigmine restored the nerve-evoked contractions which had been depressed by hexamethonium, mecamylamine or pempidine. When ganglion block was produced by hexamethonium, as in Fig. 2a, the anticholinesterase drugs also restored contractions produced by the ganglion stimulant drugs. Fig. 2a illustrates this effect of physostigmine and DMPP. However, when mecamylamine or pempidine were the ganglion blocking agents used, the anticholinesterase drugs did not restore responses to the ganglion stimulant drugs. Fig. 2b illustrates the effects of mecamylamine, physostigmine and DMPP. These results clearly demonstrate a



difference between the mechanisms of action of hexamethonium on the one hand, and mecamlamine and pempidine on the other, although precisely what the difference is has not yet been determined. At least two cholinergic junctions appear to be involved in contractions of the oesophagus evoked by vagus stimulation—the vagal ganglia and the neuro-effector junction—and the experiments give no information as to whether the effect of the anticholinesterase drugs in conjunction with ganglion blocking drugs is exerted at one or at both of these junctions.

Hemicholinium, which has been shown to inhibit the synthesis of acetylcholine by mammalian nervous tissue (Schueler, 1960), depressed the contractions of the oesophagus evoked by nerve stimulation in concentrations of 5-10  $\mu\text{g./ml.}$ , and the contractions were characteristically restored by the addition of choline (50  $\mu\text{g./ml.}$ ). Fig. 3 illustrates the effects of hemicholinium. The first panel of Fig. 3 shows control responses to acetylcholine and to nicotine. At the height of the transmission failure produced by hemicholinium, the response to acetylcholine was reduced by about 40 per cent while the response to nicotine was completely abolished. The relatively small depression in acetylcholine response may reflect a weak atropine-like action of hemicholinium, or may simply be due to fall-off of response with time. The complete block of the response to nicotine might be due to a ganglion blocking action of hemicholinium of the competitive type, but this is unlikely as hemicholinium was more effective than hexamethonium in blocking contractions to nerve stimulation. Furthermore choline, added in the continued presence of hemicholinium, restored both the contractions produced by nerve stimulation and those produced by nicotine, and these are characteristic responses occurring in the presence of a drug which inhibits acetylcholine synthesis. It therefore appears that the abolition of the response to nicotine can be explained by hemicholinium inhibiting acetylcholine synthesis at post-ganglionic cholinergic nerve endings. Apart from the first one, choline did not increase responses to acetylcholine. Potentiation of the first response to acetylcholine after the addition of choline has also been observed in mammalian skeletal muscle (Bowman and Rand, 1961). Similar results with hemicholinium, acetylcholine and nerve stimulation were obtained when the oesophagus was stimulated coaxially in the presence of hexamethonium, confirming that hemicholinium causes transmission failure at post-ganglionic nerve endings, in addition to its probable similar action at ganglionic synapses. As at other cholinergic junctions, transmission failure produced by hemicholinium was more pronounced and more rapid in onset, the higher the frequency of stimulation.

Morphine (1  $\mu\text{g./ml.}$ ), which has been shown to inhibit the release of acetylcholine from post-ganglionic cholinergic nerve endings in the guinea-pig ileum (Paton, 1957), also depressed contractions of the chick oesophagus evoked by nerve stimulation or by coaxial stimulation.

Responses to added acetylcholine were not depressed by morphine confirming that the effect of morphine was exerted on nervous structures rather than on the smooth muscle itself.

The chick gut has been shown to contain 5-hydroxytryptamine (Erspamer, 1954), and this amine was found to be highly potent in producing contractions of the oesophagus. Effective concentrations of 5-HT were of the order of 2-5 nanog./ml. and the contractions were

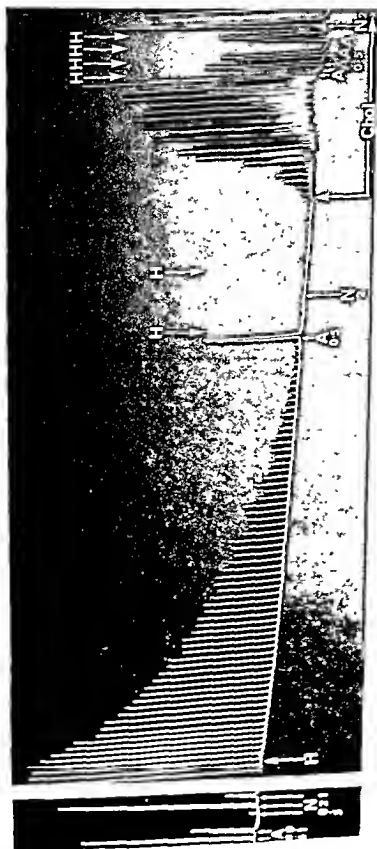


FIGURE 3

Isolated oesophagus. First panel: control responses to  $0.5$  and  $1 \mu\text{g}$  ml. acetylcholine (A) and  $0.2$  and  $1 \mu\text{g}$  ml. nicotine (N) left in contact with the tissue for 30 sec. Second panel: labelled contractions are responses to  $0.2$  and  $1 \mu\text{g}$  ml. of 5-HT. At  $10$  sec.  $1 \mu\text{g}$  ml. of hemicholinium was added and contractions ceased with the tissue. The hemicholinium was replaced each time it was washed out along with another drug. At A,  $0.5 \mu\text{g}$  ml. acetylcholine and at N,  $2 \mu\text{g}$  ml. nicotine was added to the bath and left in contact with the tissue for 40 sec. Choline (Chol.  $30 \mu\text{g}$  ml.) was added to the reservoir of Krebs' solution during the period indicated.

completely abolished in the presence of bromolyscrgic acid diethylamide ( $1 \mu\text{g./ml.}$ ), a specific antagonist of 5-HT. It has been suggested (Day and Vane, 1963; Brownlee and Johnson, 1963; Johnson, 1964) that 5-HT acts indirectly in mammalian gut through the release of acetylcholine from nervous structures. This could not be confirmed for the chick oesophagus; atropine, an antagonist of acetylcholine, and morphine, which prevents the release of acetylcholine from nervous structures, had little effect on the responses to 5-HT. It therefore appears that in chick oesophagus 5-HT acts mainly by a direct action on the smooth muscle. It has been postulated that in the guinea-pig,

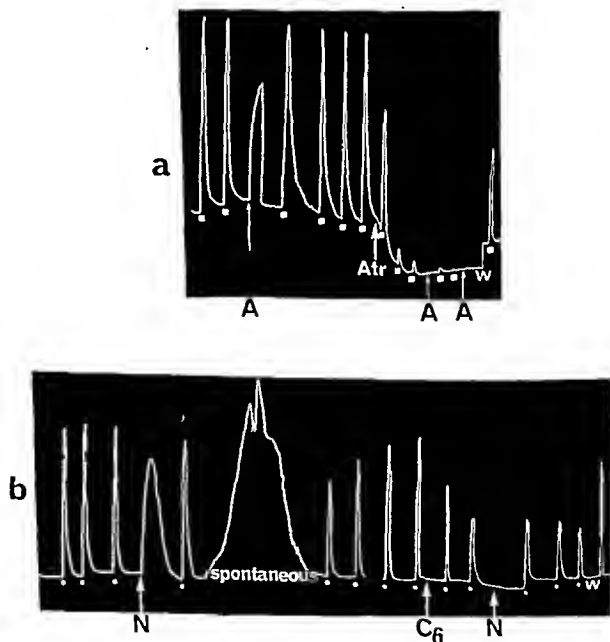
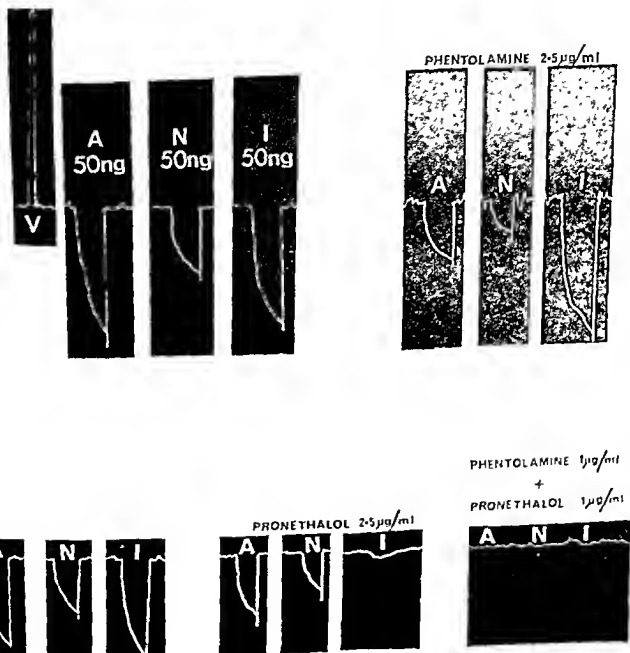


FIG. 4. Partially expanded isolated crop. The white dots mark responses to nerve stimulation (5/sec. for 10 sec., every 2 min. except when acetylcholine or nicotine were added). At A, acetylcholine ( $0.1 \mu\text{g./ml.}$ ) and at N, nicotine ( $10 \mu\text{g./ml.}$ ) were added to the bath, the first dose of each being left in contact with the tissue for 30 sec. At Atr, atropine ( $0.01 \mu\text{g./ml.}$ ) and at C<sub>6</sub>, hexamethonium ( $4 \mu\text{g./ml.}$ ) were added to the bath; each was washed out at W.

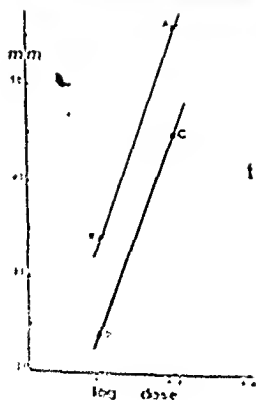
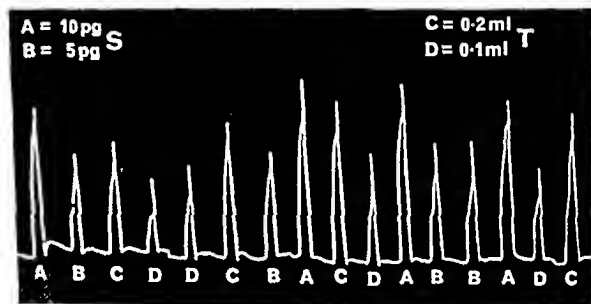
5-HT plays a part in the initiation and control of peristaltic activity of the gut (Bülbring and Lin, 1958; Bülbring and Crema, 1959*a* and *b*). In view of the presence of 5-HT in the chick gut and its high potency, it may be that the amine also plays a role in gut activity in this species and experiments are being designed to test this possibility.

The oesophagus also contracted strongly in response to histamine ( $0.1-0.5 \mu\text{g./ml.}$ ) and these responses were blocked by the antihistamic mepyramine ( $0.01 \mu\text{g./ml.}$ ). The potency of histamine was found to be considerably less during the winter months.



*Crop.*

Experiments on the crop showed that it responded to pharmacological agents and to nerve stimulation in a similar way to the upper oesophagus, and Figs. 4 and 5 illustrate some typical responses. Fig. 4a



$$T = 35.3 \text{ pg/ml}$$

$$(89.08 - 112.3)$$

$$\text{true value of } T = 32 \text{ pg/ml}$$

$$\frac{s}{b} = 0.053$$

illustrates the effect of atropine in blocking contractions of the crop produced by nerve stimulation and by added acetylcholine, and Fig. 4*b* illustrates the effects of hexamethonium in blocking contractions produced by nicotine and depressing those produced by nerve stimulation. The expanded crops occasionally contracted spontaneously, and a spontaneous contraction is illustrated in Fig. 4*b*. Fig. 5 illustrates responses of the crop to sympathomimetic amines and the effects of both  $\alpha$ - and  $\beta$ -receptor anti-adrenaline drugs. (—)-adrenaline, — -noradrenaline and (—)-isopropylnoradrenaline caused dilatation of the partially expanded crop, adrenaline and isopropylnoradrenaline being about equi-potent and noradrenaline less so. The  $\alpha$ -receptor blocking drug phentolamine reduced but did not abolish responses to adrenaline and noradrenaline and was without effect on responses to isopropylnoradrenaline. The  $\beta$ -receptor blocking drug pronethalol reduced the responses to adrenaline and noradrenaline and abolished those to isopropylnoradrenaline. A mixture of both blocking drugs completely abolished the responses to all three sympathomimetic amines. The blocking drugs did not affect responses to vagal stimulation. These results are essentially similar to those obtained with the upper oesophagus, some of which have been previously reported (Bowman and Everett, 1964).

#### *Assay of 5-hydroxytryptamine*

During the months of May to September, the crop proved to be exceptionally sensitive to 5-HT and transverse strips of crop from starved chicks were used during the period to develop a sensitive biological assay for this amine (Everett, 1964). Fig. 6 illustrates an assay carried out in this way. Some strips were even more sensitive and responded to  $10^{-12}$  g./ml. (1 picogram) of 5-HT. The method is suitable for the assay of 5-HT in urine and peritoneal fluid and probably in other body fluids also. It can be carried out in the presence of sufficient atropine and mepyramine to block responses to acetylcholine and histamine selectively, and the preparation is in any case insensitive to bradykinin.

Weak solutions of 5-HT in Krebs' solution rapidly lose activity, and it may be necessary to make up the standard solutions in slightly acid distilled water so that the required dose is contained in 0.1 ml. Immediately before addition to the preparation, 0.1 ml. of standard or test solution is added to 0.9 ml. of Krebs' solution and the total volume of 1 ml. is then applied to the tissue.

During the autumn and winter months, the sensitivity of the crop to 5-HT diminished markedly; during December and January some preparations were completely insensitive and others responded only weakly to concentrations as high as 1 mg./ml.



### Discussion

Although clearly more information is necessary, the preliminary pharmacological experiments described suggest that the innervation of the chick oesophagus and crop is essentially similar to that of the mammalian alimentary canal and that the same neurotransmitters are involved.

The two main points arising from this work are as follows. Firstly, the oesophagus preparation described provides a useful isolated tissue for studying the action of drugs, both for research workers and for undergraduate students. Its particular advantages for student classes lie in the ease of setting it up, the clear-cut nature of its responses and its cheapness. It has already been incorporated into our practical classes and we are finding it successful. Preparations taken from birds older than 7 days are less useful. Rand and Stafford (1964) studied oesophagus preparations from various adult species including the domestic fowl which responded relatively poorly to drugs and only to high concentrations which were then difficult to wash out. They attributed this to a dense connective tissue barrier which retarded diffusion. Preparations from chicks under 7 days old are relatively free from connective tissues. Secondly, during the summer months, the crop strip provides a useful assay preparation for 5-HT which is more sensitive and easier to set up than other available methods.

### Acknowledgements

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# PHARMACOLOGICAL RESPONSES OF THE SMOOTH MUSCLE OF THE CHICK AMNION

A. W. CUTHBERT

*University Department of Pharmacology, Cambridge*

## *Synopsis*

EVIDENCE is presented to show that the increased muscular activity of the amnion resulting from the application of the cholinesterase inhibitor, eserine, results from the accumulation of endogenous acetylcholine within the smooth muscle layer. The cross-figures are considered to be the sites for the most intense production of endogenous acetylcholine and the sites from which spontaneous contractions arise. Thus the cross-figures act as pacemakers for myogenic amniotic activity. It seems probable that the endogenous acetylcholine does not act at the muscarinic receptors in the cell membrane which are available to externally applied acetylcholine. A possible intracellular mode of action for endogenous acetylcholine is suggested.

## *Introduction*

In this paper the actions of some drugs, mainly anticholinesterases, on the smooth muscle of the chick amnion are considered. Results from this pharmacological study suggest a chemical basis for the spontaneous mechanical activity exhibited by this extra embryonic structure. Before the results with anticholinesterase drugs are discussed it is necessary first to review the structure and functioning of the amnion together with the distribution of the enzyme cholinesterase within the amniotic membrane.

## *Experimental*

### *Structure and Function of the Amnion*

The amniotic membrane forms a temporary living appendage to the developing chick embryo and consists, at least in the early stages, of two single layers of cells. The inner layer of epithelial cells are derived from ectoderm, whereas the outer single layer of smooth muscle cells is of mesodermal origin. It is this layer of smooth muscle cells which endows the amnion with its ability to contract spontaneously and so prevent adhesions forming during the early stages of embryonic development. The frequency of the spontaneous contractions varies during incubation

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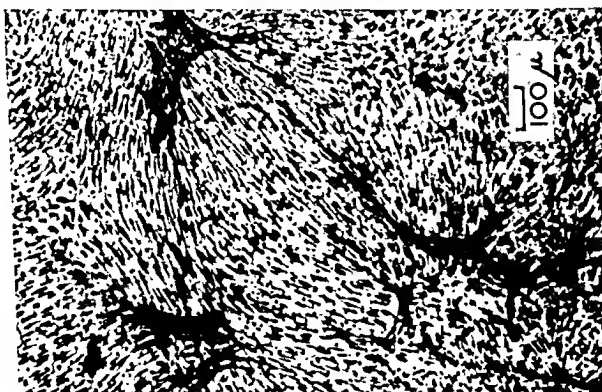
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Smooth muscle of a 6 day amniotic membrane (head-fold region) stained with iron-haematoxylin.

as was shown by Kuo (1932). Spontaneous activity appears first at about 4 days, is maximal around 10-11 days and falls to zero, or a low value, after 2 weeks.

The arrangement of the smooth muscle fibres within the mesodermal layer is unusual. Some fibres are arranged in bands running radially from the umbilicus, whereas on the dorsal aspect of the amniotic sac double-layered stars or crosses of smooth muscle cells are seen. These will be referred to throughout as cross-fingers, and are illustrated in Plate 17. The distribution of these cross-figures has been described by Bautzmann and Schroder (1953). They occur principally on the dorsal aspect of the sac with local concentrations at the head and tail-fold regions. There is a remarkable correlation between the formation and distribution of cross-figures and the spontaneous activity of the amnion.

- (i) Cross-figures appear first at 4 days, the time at which spontaneous activity commences.
- (ii) Cross-figures are most numerous in the head-fold region, then at the tail-fold. Spontaneous contractions usually arise at the head-fold and pass caudally, although occasional contractions arise at the tail-fold and pass in the reverse direction.
- (iii) The density of the cross-figures is greatest at 10 days, whereas by 14 days almost all the cross-figures have disappeared (Clements and Ferguson, 1951). These times correspond with the times of maximal activity and of cessation of activity respectively.

As long ago as 1908, Verzar suggested that the cross-figures acted as pacemakers for the amnion, rather in the same way as did nodal tissue in the heart. In the heart the action potentials recorded from nodal tissue differ from those recorded from the ventricle in that the former show an initial phase of slow depolarisation, the prepotential. Action potentials from the amnion, like those of cardiac tissue are of the plateau type and some show a well defined prepotential (Cuthbert, 1964). It would be interesting to know if amnion action potentials showing a prepotential were recorded only from cross-figures.

Histological studies by many workers have failed to reveal any nerve fibres in amnion smooth muscle. The most recent study by Evans and Evans (1964) using the electron microscope confirms the absence of innervation in this structure. Thus the effects of drugs on the contractility of the amnion must be due to a direct effect on the smooth muscle cells and not mediated through a nervous mechanism.

#### *Cholinesterase Distribution in the Amnion*

The histochemical distribution of cholinesterase in the amnion can be demonstrated by either the method of Koelle (1951) or that of Lewis (1958). In both methods the tissue is incubated with a substrate which

is hydrolysed by the enzyme and one product of the hydrolysis is visualised by forming a coloured compound with suitable reagents. Acetylthiocholine and butyrylthiocholine have been used as substrates using the Koelle technique. The resulting thiocholine is visualised by forming a copper complex and converting the latter to black copper sulphide. With the Lewis method the substrate is  $\alpha$ -naphthyl acetate. The resulting  $\alpha$ -naphthol is diazotised to give a red brown azo-dye. Suitable cholinesterase inhibitors were used with each method to check that the staining reaction did not occur when the hydrolysis of the substrate by the enzyme was prevented.

Cholinesterase distribution in the amnion has the following characteristics (Cuthbert, 1963):

- (i) the enzyme is confined to the smooth muscle layer, the epithelial cells failing to show any staining reaction;
- (ii) the enzyme appears to be located intracellularly;
- (iii) the cross-figures show the greatest density of staining, indicative of a higher concentration of enzyme within these regions;
- (iv) in young membranes (6 days' incubation) only the cross-figures exhibit the stain;
- (v) at the time when the cross-figures are most numerous (9-10 days) they become interwoven and interconnected with bands of muscle fibres; these bands also show a greater density of stain than the surrounding muscle sheet.

### Discussion

Does the cholinesterase of the amnion have a physiological role? Other examples, such as the neuromuscular junction in skeletal muscle, are known where cholinesterase occurs in high concentration and plays a physiological role in the removal of acetylcholine. Acetylcholine can be detected in extracts of amnion tissue (Cuthbert, 1963) and must be derived from either the epithelial or muscular tissue as nervous elements are absent. In 1956, Jones, Featherstone and Bonting reported that chick embryo intestine grown in tissue culture in the presence of acetylcholine produced more cholinesterase than in the absence of acetylcholine. Thus the substrate induced the formation of enzyme. The enzyme of the chick intestine is identical with that of the amnion (Blaber and Cuthbert, 1962). It is suggested that the phenomenon reported by Jones *et al.* (1956) is operative in the amnion and that the greater concentration of cholinesterase in the cross-figures results from a greater concentration of endogenous acetylcholine. In the next section the case for the involvement of acetylcholine in the generation of spontaneous mechanical activity is made.

### *The Actions of Anticholinesterases*

Anticholinesterase drugs, by inhibiting the cholinesterase, should allow the accumulation of endogenous acetylcholine. The latter, in



turn, should increase the myogenic activity. This argument is valid only if the anticholinesterase drugs themselves can be shown to exert no direct stimulant action on the amnion smooth muscle.

Some characteristics of the responses of the amnion to anticholinesterases are as follows and summarise two reports by Cuthbert (1962 and 1963).

- (i) The amnion responds to the anticholinesterase drug eserine but not to neostigmine. The former drug being a tertiary amine is able to penetrate the cell membrane and inhibit the intracellular cholinesterase, whereas the quaternary compound neostigmine, is unable to do this. Both eserine and neostigmine are equally effective in inhibiting the cholinesterase of an amnion homogenate. These results are therefore complementary to the histochemical findings and suggest that if eserine acts by allowing the accumulation of endogenous acetylcholine then this must occur intracellularly. Normally in avian smooth muscle both eserine and neostigmine will potentiate the response to cholinergic nerve stimulation indicating that at least part of the cholinesterase is extracellular (see Everett, 1966). It is of interest that the amnion smooth muscle, which is not continually bombarded with the cholinergic transmitter, does not have extracellular cholinesterase. Two irreversible anticholinesterases DFP and mipafox produce results like those obtained with eserine and neostigmine respectively. Again only the former compound, DFP, penetrates the cell membrane rapidly, although both compounds are equipotent anticholinesterases against the isolated enzyme.
- (ii) Old membranes (16-17 days) in which spontaneous activity has ceased, fail to respond to eserine although they do so to acetylcholine. At this time the cross-figures have disappeared and presumably endogenous acetylcholine production has ceased. The lack of effect of eserine on old membranes in which the musculature remains responsive to acetylcholine suggests that eserine does not act directly.
- (iii) Membranes in which the cholinesterase has been irreversibly inhibited by DFP fail to respond further to eserine. This again demonstrates the lack of a direct effect of eserine.
- (iv) The response to eserine is temperature dependent as are the spontaneous contractions. Both the eserine response and the spontaneous activity disappear at 30°C. and both are maximal around 38°C. These results are expected if both the eserine response and the spontaneous contractions are dependent upon the production of endogenous acetylcholine by enzymic processes.
- (v) The response to eserine is not blocked by atropine, whereas that to acetylcholine is blocked. This suggests that the

muscarinic acetylcholine receptors in the cell membrane, which are available to externally applied acetylcholine, are not available to endogenous acetylcholine. One possibility is that endogenous acetylcholine may trigger activity by liberating calcium from some bound form within the cell. Calcium is known to be involved in excitation contraction coupling and the response of the amnion to eserine is calcium dependent.

The results presented in this paper contain many assumptions and some of the data may be capable of alternative explanation, but this author feels there is sufficient evidence to involve acetylcholine in the spontaneous activity of the smooth muscle of the chick amnion.

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## CATECHOLAMINES IN THE CHICK

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University of London**Synopsis*

THE ABILITY of guanethidine to deplete the stores of catecholamines in the brain of the young chick is investigated. As the age of the chick increases, the potency of the guanethidine decreases until the drug is no longer active, suggesting that a barrier to its entry into the brain may have developed.

The proportion of adrenaline present in the total catecholamine content of the chick hearts and brains is also studied since it was found to be much higher than in corresponding mammalian tissues. An attempt is made to correlate the adrenaline content of hearts, brains and adrenal glands with their weights. The depletion of the adrenaline and *nor*-adrenaline contents of hearts and brains by guanethidine is also studied.

The possibility that adrenaline could be the sympathetic transmitter in the chicken is discussed.

*Introduction*

It has been known for some time that at hatching the chick blood-brain barrier to certain substances is incompletely developed. It has been shown (Waelseh, 1955) that the rate of uptake of labelled chloride into chick brain is considerably slowed between 16 and 28 days after hatching and Key and Marley (1962) showed that sympathomimetic amines which produce E.E.G. arousal in young chicks fail to do so after they are about 28 days old. It has also been observed that intravenous injection of adrenaline into the young chick produces a state resembling sleep (Zaimis, 1960), but to produce a comparable state in the mammal requires intraventricular injection (Feldberg and Sherwood, 1954). If this permeability of the blood-brain barrier in the young chick were a general phenomenon it would be a most useful tool for investigating the effects of drugs on the CNS. For example, the adrenergic neurone blocking drug guanethidine also has the property of

depleting mammalian peripheral tissues of their catecholamine content, but it does not alter the amine content of the brain or adrenal medulla. This difference as regards the brain has been attributed to the inability of guanethidine to pass the blood-brain barrier. It was decided to test this using the chick.

### *Results and discussion*

In these, and all subsequent experiments male chicks of the New Silver Link strain were used. The results we obtained are shown graphically in Fig. 1.

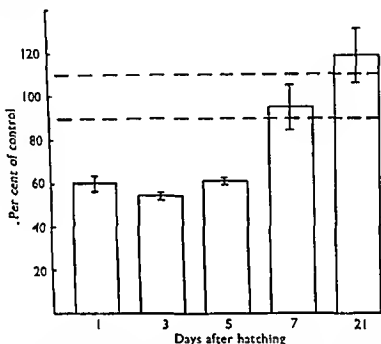


FIG. 1. Effect of guanethidine (100 mg./kg. s.c.) on chicken brain catecholamine concentration expressed as *nor*-adrenaline at various times after hatching.

*Vertical bars:* standard error.

*Broken lines:* standard error of control mean.

A single dose of guanethidine, 10 mg./kg. was injected s.c. into chicks at various times after hatching and the animals killed by decapitation 18 hr. later. The brains were removed and assayed fluorimetrically for catecholamine content in terms of *nor*-adrenaline. Guanethidine induced a depletion of the amine up to 5 days, but between 5 and 7 days there was an abrupt change, the depletion being abolished. Hearts taken from these animals were still depleted at 21 days so this change is presumably due to the establishment of a blood-brain barrier to the drug. It is of interest that the time at which this change occurs is not constant; it may be any time between 1 and 4 weeks after hatching. Reserpine is another drug which depletes tissue amines and is much more potent in this respect than guanethidine. However, for a given dose of reserpine in mammals the brain and the adrenal glands are less affected than are other catecholamine containing tissues. Collins in our laboratory using single small doses of reserpine (0.5

mg./kg.) in chicks at various times after hatching and assaying the brains and adrenal glands found the pattern shown in Fig. 2. The 50 per cent depletion of brain amines produced up to 10 days by reserpine is abolished in the older chicks although at this time there is a 77 per cent depletion in the heart. This suggests that the relative resistance of mammalian brain amines to reserpine-induced depletion is due to some interference by the blood-brain barrier with the entry of reserpine to the brain, rather than to a greater resistance to depletion of brain catecholamine stores. In fact, in young chicks the brain and heart are

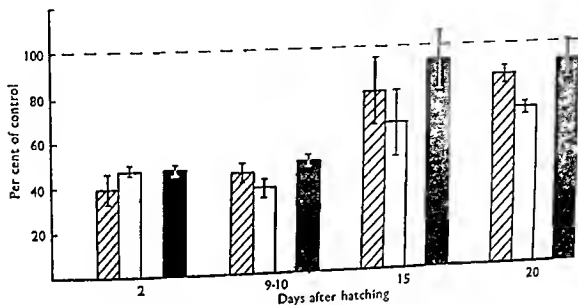


FIG. 2. Effect of reserpine (0.5 mg./kg. s.c.) on chicken brain and adrenal catecholamine concentration at various times after hatching.

*Hatched columns:* noradrenaline } in adrenal glands.  
*Open columns:* adrenaline  
*Closed columns:* total amine as noradrenaline in brain.  
*Vertical bars:* standard error of the mean.

equally affected by the same dose of reserpine, i.e. the stores, at least at this stage, are equally susceptible. However, it is also interesting that the degree of adrenal depletion reflects that of the brain, there being very much less depletion in the older chicks. There are two possible explanations for this, firstly that the relative resistance of the adrenals to amine depletion is due to the formation of some sort of blood-adrenal barrier, analogous to the blood-brain barrier, or secondly that the depletion of the adrenals is mediated centrally, probably through the hypothalamus, for which there is evidence in some mammals (Holzbauer and Vogt, 1956). However the mechanism is still obscure and it may be possible, by the use of a wide range of depleting drugs and doses in the chick to elucidate this. The best experiment would be to test the effect of reserpine in chicks after denervation of the adrenal glands, if this were technically feasible.

While we were carrying out these experiments, it was noticed that a large proportion of the catecholamine being measured appeared to be adrenaline. Nor-adrenaline is the amine liberated from the ends of

postganglionic sympathetic nerves and is therefore the transmitter of the sympathetic nervous system and mediates normal sympathetic function. Adrenaline differs from *nor*-adrenaline in that it has a methyl group substituted on the terminal nitrogen, and it is the principal amine secreted by the adrenal medulla in response to stress—the so-called “fight or flight” reaction. In mammals, the proportion of adrenaline in tissues, other than the adrenal medulla, does not normally exceed 10 per cent of the total catecholamine content. Hearts, brains and adrenal glands of chicks were therefore assayed at various ages for both adrenaline and *nor*-adrenaline, and the results for hearts and brains are shown in Fig. 3. This shows that the concentration of adrenaline in brain is appreciable, being about 30 per cent and that the ratio of adrenaline to *nor*-adrenaline remains relatively constant as does the concentration, although the weight of the tissue changes as the animals grow. In the heart the concentration of adrenaline is considerably higher than that of *nor*-adrenaline which falls with age while that of adrenaline apparently varies but does not decrease. In the adrenal glands the total catecholamine concentration rises slightly with age from about 5 mg./g. to about 7 mg./g. but the proportion of adrenaline remains constant around 50 per cent. If these results are expressed in a different way, as in Fig. 4 it is perhaps easier to see their possible significance a little more clearly. This shows the mean weight of the tissues and their total content of adrenaline and *nor*-adrenaline. The

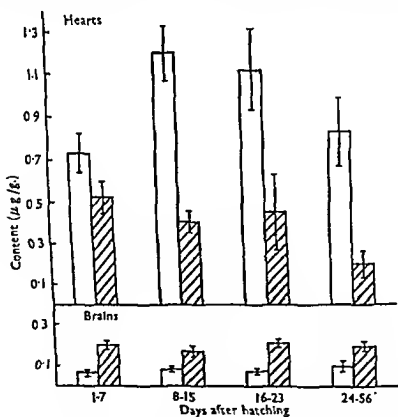


FIG. 3. Concentration of adrenaline (open columns) and *nor*-adrenaline (hatched columns) in chicken hearts and brains at different ages.

Vertical bars: standard error.

weight of the hearts increases ten-fold between 1 and 8 weeks and the content of adrenaline closely parallels this—the two curves are almost superimposable. However, the *nor*-adrenaline content only increases 4 times and the slope is much shallower. This indicates that there is a marked increase in methylation during development in these chick hearts. In brain and adrenals the picture is somewhat different. The weight of the brain doubles over the 8 weeks and so do both the adrenaline and noradrenaline contents—the curves are parallel and there is no evidence of an increase in methylation. Likewise in the adrenals, although there is a slight increase in concentration the adrenaline and *nor*-adrenaline contents increasing rather more than the adrenal weight, there is no evidence of an increase in methylation. This is a little surprising because in most other species there is an increase in methylation during early life in the adrenal medulla. However, Leibson and Stabrowski (1962), have found that an increase in methylation does occur just at hatching. The percentage of adrenaline goes up from 35 at 1 day before to 51 at 1 day after hatching. Incidentally their estimates both of total amount of catecholamines and of distribution of adrenaline and *nor*-adrenaline in chick adrenals agree very well with our own, whereas the only other estimates of young chick adrenals we can find in the literature (Shepherd and West, 1951) show a higher total amount and a higher percentage of *nor*-adrenaline. This may, however, be a strain difference or possibly an artefact due to the

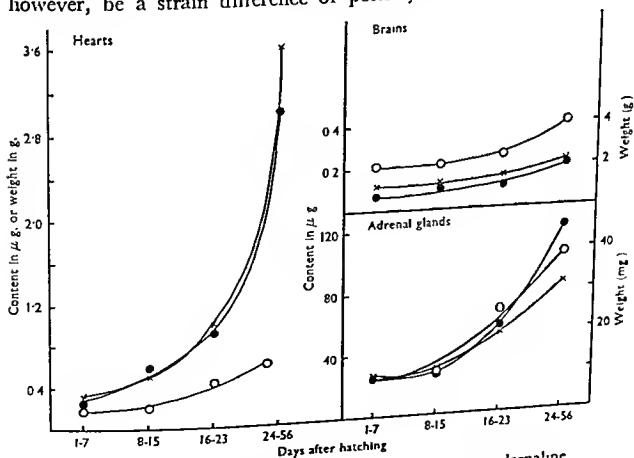


FIG. 4. Content of adrenaline  $\bullet$ — $\bullet$  or noradrenaline  $\circ$ — $\circ$ , and weight of tissue  $\times$ — $\times$  of hearts, brains and adrenal glands in chicks at various ages.

different techniques used for assay. Leibson and Stabrowski (1962) and ourselves used fluorimetry whereas Shepherd and West (1951) used bioassay. We have already found a discrepancy between catecholamine values of rat adrenal glands assayed by the two techniques and are investigating possible reasons for this.

Does the high proportion of adrenaline in the hearts have any significance? It may be relevant here to recall that frog tissues contain a very high proportion of adrenaline—in fact von Euler (1946) was unable to detect any *nor*-adrenaline in the frog heart. In the one group

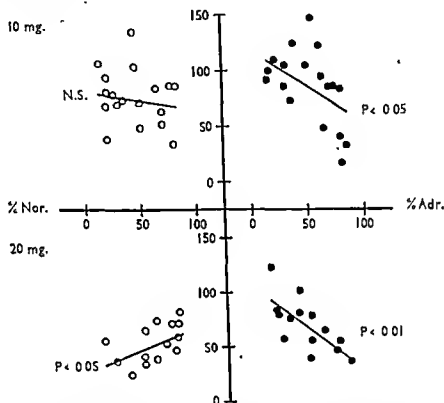


FIG. 5. Correlation between percentage of adrenaline (closed circles) or noradrenaline (open circles) before and after guanethidine (10 or 20 mg./kg. s.c.) in chick hearts and brains. Lines are "calculated lines of best fit".

of frogs that we have tested we also could not detect any *nor*-adrenaline but found a very high amount of adrenaline (about 10  $\mu\text{g./g.}$ ). von Euler considers that the sympathetic transmitter in the frog heart is adrenaline. This raises the possibility that the sympathetic transmitter in the chick heart may be, or may contain a large proportion of adrenaline. The increase in methylation in the hearts suggests that adrenaline may be the more important amine and some experiments we have done using guanethidine lend further support to this idea. These results are illustrated in Fig. 5.

The degree of adrenaline and *nor*-adrenaline depletion in hearts and brains before the blood-brain barrier is established, found after using guanethidine, was extremely variable and did not seem to correlate with dose or age. However, when the degree of depletion of either adrenaline or noradrenaline was plotted against the percentage of that amine in the tissue, then a significant correlation emerged. Where



there is a high proportion of adrenaline it is subject to depletion, but where there is only a small percentage there is no depletion and occasionally even an increase. By contrast, *nor*-adrenaline usually shows a slight depletion but it is very variable and there is little or no correlation at all and the calculated line is not significant. When the dose is increased to 20 mg./kg., the correlation for adrenaline becomes even better and the line moves downwards showing that the degree of depletion for each percentage is greater; and even at low percentages there is usually some depletion. The *nor*-adrenaline depletion now shows a correlation with the percentage of amine but in contrast to the adrenaline it is the smaller proportion of amine which is less readily depleted. That is, there may be a small labile pool and a larger stable pool for *nor*-adrenaline, whereas the adrenaline may be in a large labile pool, with only a small stable or storage pool. We believe that this would be in agreement with a functional role for adrenaline and a precursor or storage role for *nor*-adrenaline.

To conclude, we must emphasise that these results are of a preliminary nature and the suggestions we have made are to serve as a basis for further work.

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## RENAL FUNCTION IN THE FOWL

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THE PRINCIPAL anatomical features of the venous system of the fowl kidney are described. The evidence for the existence of a functional renal portal system is reviewed and the use that may be made of this system to determine excretory mechanisms is discussed. Some effects of experimental modifications of the vascular supply on renal function are described.

*Introduction*

One of the most characteristic features of the avian kidney is its venous system (Fig. 1). There is a principal renal vein draining the middle and posterior lobes in a way comparable to that of the mammalian renal vein and, according to some authors, a number of vessels draining the anterior lobe (Sperber, 1948). In addition, the posterior and middle lobes and probably the anterior lobe receive an additional supply to the peritubular capillaries from the branches of the renal portal vein. This vein is unique in that it affords a direct connection between the hepatic and renal circulations via the coccygeo-mesenteric vein but there is an important difference between these two portal systems.

In the hepatic portal system the vein breaks down completely to a second capillary network, whereas in the renal portal system there is a very large channel which allows blood entering the portal vein, from the legs and the coccygeal region, to return directly to the inferior vena cava, and so to the heart, without first traversing the peritubular capillaries. These capillaries offer an alternative route, the functional nature of which was not clear until the experiments of Sperber (1948). He showed, by catheterisation of both ureters followed by the injection of phenol red into the muscle of each leg in turn, that the kidney on the injected side excreted a greater quantity of phenol red than the kidney on the opposite side. It was concluded that this excess had been transported to the tubules through the portal capillaries since both kidneys excreted similar quantities when phenol red was injected into the pectoral muscle.

*Experimental*

A similar response to that described by Sperber (1948) to *p*-amino-hippurate (PAH) may be seen in Fig. 2 in which the further point is made that unilateral infusion of inulin into the tibial vein does not result in any increase in the glomerular filtration rate. Hence it may be concluded that the portal capillaries do not reach the glomeruli, a point that has also been made on histological grounds by Spanner (1925). Uric acid, also secreted by the tubules, similarly shows differential excretion following unilateral injection (Fig. 3), and so too do a number of unrelated substances of pharmacological interest, c.g. tetraethyl ammonium, histamine and penicillin. In fact, Sperber's technique has probably been used more by pharmacologists than by those with an interest in avian physiology and it might be appropriate to point out some of the limitations of this method of establishing whether or not a particular substance is subject to tubular secretion.

The normal procedure for this is to make a comparison of the renal clearance of the particular substance with that of inulin and an obvious advantage of Sperber's technique is that it obviates the need for intravenous infusions and plasma analysis; only the urinary excretion is required. On the other hand, if the tubular maximum for the substance

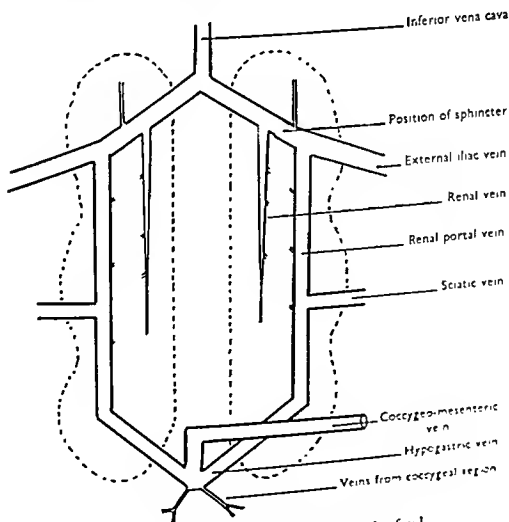


FIG. 1. The principal renal veins of the fowl

under investigation is exceeded then no differential excretion can be observed even if the clearance is still well above filtration rate.

This can be seen even with PAH which has a high tubular maximum (Table 1). The clearance on the infused side under conditions of a low tubular load clearly was overestimated since there was no means of determining the effective concentration of PAH in the blood. With the high load this error, although still present, would be reduced. Although the differential excretion was abolished there was still positive evidence of tubular secretion from the clearances, which remained well above the filtration rate.

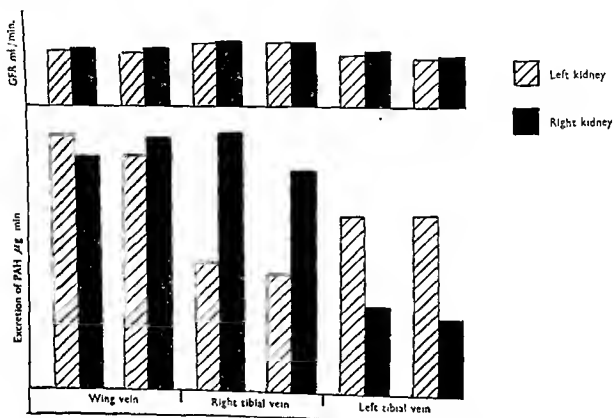


FIG. 2. Excretion of PAH and filtration rate following infusion at different sites to demonstrate the renal portal supply to the tubules.

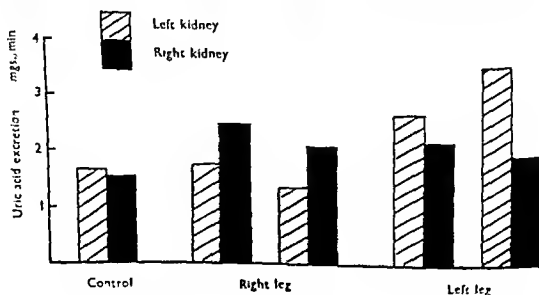


FIG. 3. Excretion of uric acid through the renal portal system of the fowl.

With substances with a very low tubular maximum a differential rate of excretion might never be observed. This was true of creatine which, nevertheless, had a clearance of approximately twice the filtration rate.

Another disadvantage of this technique was observed when urea was infused into the tibial vein on one side. An excess excretion occurred on the same side and this might have been taken as evidence of secretion

TABLE 1

*The renal clearance and excretion of p-amino-hippurate (PAH) following infusion at different rates (L and R=left and right kidneys)*

Site of infusion	Inulin clearance ml./min.		PAH			
			Excretion mg./min.		Clearance ml./min.	
	L	R	L	R	L	R
(a) The infusion of small quantities of PAH						
Wing	2.3	2.6	0.43	0.39	55.0	50.0
	2.2	2.4	0.20	0.21	49.0	53.0
Right leg	2.6	2.7	0.11	0.22	42.5	84.0
	2.7	2.7	0.10	0.19	55.5	104.0
Left leg	2.1	2.3	0.15	0.08	18.8	9.4
	2.0	2.1	0.15	0.07	84.5	36.6
(b) The infusion of large quantities of PAH						
Wing	3.4	3.1	16.10	15.80	4.48	4.40
	3.2	3.6	13.75	13.50	5.30	5.10
Right leg	2.9	2.7	11.70	11.60	6.15	6.10
	2.8	2.8	11.25	11.25	6.25	6.25
Left leg	2.8	2.7	11.0	10.30	6.90	6.46
	2.2	2.2	10.50	10.10	6.80	6.50

despite other evidence to the contrary (Owen and Robinson, 1964). However, on examination of the data (Sykes, 1962) it was clear that there had been a unilateral diuresis and the concentration was *not* invariably higher on the infused side. The observed result could be interpreted in terms of an altered reabsorption gradient for urea which resulted in the urea, now retained in the tubule, acting as an osmotic diuretic. Whatever the explanation, this example serves to show that the total excretion rates may not be enough to establish a secretory mechanism unless the urine volumes are similar on both sides.

relaxed by atropine and adrenaline (after acetylcholine). Large doses of atropine *in vivo* were shown to reduce very drastically a differential excretion of PAH which had been established.

It has been possible to confirm this effect of atropine, but only with doses as large as 10 mg., and clearly such doses rule out any specificity of site of action. Occasionally no change in the excretion rate was observed and at other times the excretion rate from both kidneys was depressed although the differential was still present. This may be interpreted as showing that the sphincter on both sides was initially contracted, thus allowing blood to traverse the portal capillaries. The effect of the atropine was to relax both sphincters but some blood nevertheless continued to flow through the portal system as shown by the excess excretion on the injected side.

The effect of atropine was abolished by carbachol, i.e. the differential excretion was restored, but again using doses well above what is generally considered to be physiological.

In other experiments it has been possible to tie off the external iliac vein at the site of the sphincter and this procedure resulted in no rise in the differential excretion rate that had already been established. It might be concluded from this that the sphincter was completely closed at the beginning otherwise total ligation might have brought about a further increase in the blood flow to the tubules. On the other hand, the venous return from the legs might have been diverted through the hepatic portal system so as to maintain the same effective renal blood flow throughout.

The existence of these alternative routes for venous flow suggests that the avian kidney might be less affected by major circulatory changes than that of mammals. By occluding either the inferior vena cava or the hepatic portal vein it should be possible to make very considerable increases in the potential blood supply to the tubules and to see how far these alterations affect renal function.

Occlusion of the hepatic portal vein where it enters the liver was invariably fatal in chronic experiments and no measurements were made under these conditions. It was possible, however, to occlude this vein for short periods and to measure filtration rate and plasma flow by the usual clearance procedures (Sykes, 1960).

A typical experiment is given in Fig. 4. Urine flow was maintained (since a diuretic was given), but there was a moderate fall in the filtration rate and in the plasma flow. The latter was surprising since it might have been expected to increase if any of the diverted blood passed through the peritubular capillaries.

Only when the coecygeo-mesenteric vein was occluded simultaneously with the hepatic portal vein did renal function become depressed and this might be explained in terms of a reduced arterial blood pressure brought about by a sudden fall in the venous return to the heart.

Chronic occlusion of the inferior vena cava resulted in no apparent change in renal, or any other, function of the fowl. The operation was performed before maturity but subsequently all eight birds laid normally.

The birds were not examined *post mortem*, but it is likely that a collateral circulation developed as described by Clarkson and Richards (1965) in similar work on the turkey reported at this Symposium.

In the acute preparation, after occlusion of the inferior vena cava there was an immediate fall in urine flow sometimes amounting to anuria. This was accompanied by a fall in filtration rate and in renal plasma flow, but it was a transient phase and there was complete recovery sometimes within 15 min. Although only a few figures have been obtained so far, it appears that there is no large increase in plasma flow despite re-routing a major part of the total venous return through the renal circulation. This conclusion, together with the similar result of hepatic portal occlusion above, lends weight to the suggestion made by Akester (1964) that blood flow through the renal portal capillaries may be controlled more by vasomotor mechanisms within the kidney lobes than by the renal sphincter.

The importance of the sphincter, as Akester implies, may lie in diverting blood into the hepatic portal circulation but so far the functional significance of the connection afforded by the coccygeo-mesenteric vein has remained obscure.

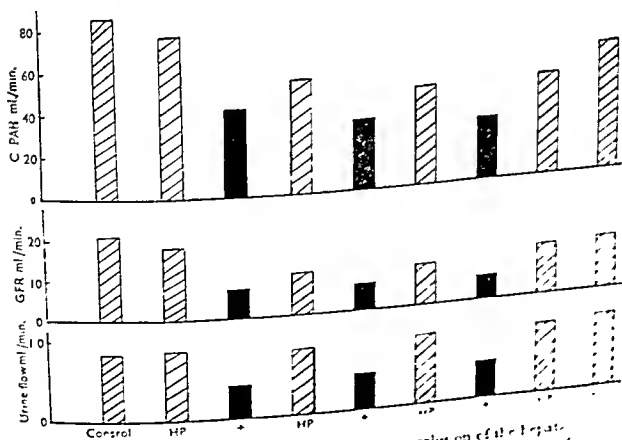


FIG. 4. Changes in renal function following occlusion of the hepatic portal vein with (—) or without HP; simultaneous occlusion of the coccygeo-mesenteric vein.

It is possible that the flow in this vessel might at times be directed towards the kidney and this would allow products from the rectum and the thick segments of the caeca to enter the renal circulation rather than the hepatic. That this route is available was demonstrated by a variation of Sperber's technique. The hypogastric vein on the right side, which is accessible for about 1 cm. in cocks and non-laying hens, was tied off so that any blood entering the renal portal circulation at this point would be available to only the left kidney. There were no apparent effects of this operation itself upon renal function.

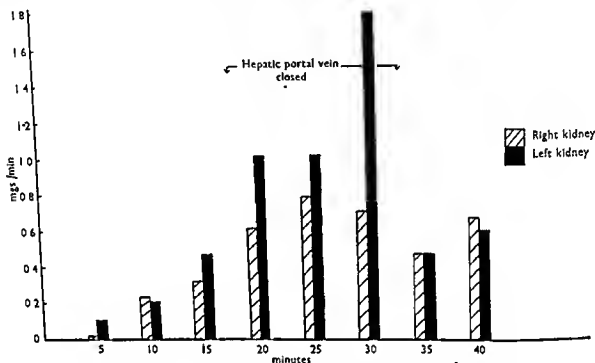


FIG. 5. The differential excretion of PAH following infusion in a mesenteric vein. R. hypogastric vein occluded.

A cannula was inserted into a small mesenteric vein and provision made for the occlusion of the hepatic portal vein as required.

The infused PAH could obviously pass towards the liver or the kidney and there is some evidence that occasionally it was to the latter. Thus in Fig. 5 a differential in favour of the left kidney was seen in periods 1 and 3 and, as might be expected, became pronounced when the hepatic portal vein was closed. Under these conditions a portion at least of the normal hepatic flow must have entered the peritubular capillaries but whether this can occur under more physiological conditions remains to be determined.

#### Acknowledgement

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## LIVER BLOOD FLOW IN THE TURKEY

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*Department of Parasitology and Entomology, Liverpool School of Tropical Medicine and the Department of Physiology, University of Liverpool**Synopsis*

THE TOTAL hepatic blood flow of unanaesthetised turkeys is measured by estimating the clearance of bromsulphthalein after a single intravenous injection at a time dynamically equivalent to continuous infusion conditions at equilibrium. Components of clearance—hepatic blood flow and extraction ratio—are then derived.

The results in normal turkeys and in birds with ligatures on either the posterior vena cava or the coccygeo-mesenteric vein are given.

The mean hepatic blood flow in 16 normal birds was 44.2 ml./kg./min., and in 7 birds after ligation of the coccygeo-mesenteric vein 40.3 ml./kg./min. Ligation of the posterior vena cava usually increased the liver blood flow considerably but the results were variable.

It is suggested that blood may flow in either direction in the coccygeo-mesenteric vein depending on the state of the renal portal sphincter (valve), thus increasing or decreasing the hepatic venous supply.

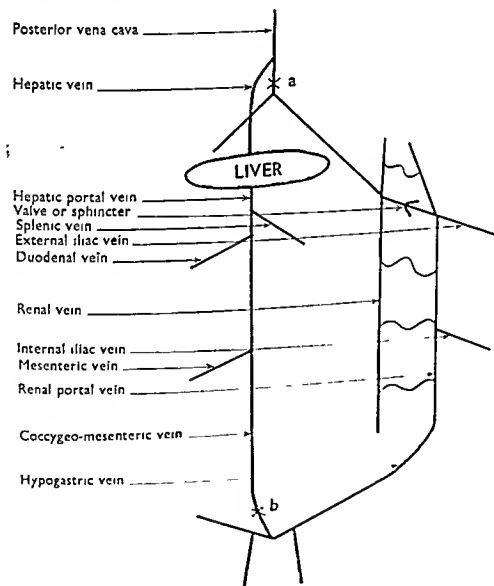
*Introduction*

The blood supply of the liver of the turkey consists of the small hepatic arteries and the hepatic portal veins (Clarkson, 1961a). The left portal vein is small and drains the gizzard and proventriculus. The right hepatic portal vein is large and receives blood from the splanchnic area including main branches from the duodenum, small intestine and the spleen. This is similar to the mammal but in addition the vein continues posteriorly as the coccygeo-mesenteric vein and connects by a venous arch to the renal portal venous system via the hypogastric veins. This system receives blood from the legs via the iliac veins and connects with the posterior vena cava. On each external iliac vein there is a muscular "valve" or sphincter (Fig. 1). The sinusoids of the liver are, therefore, directly connected to the posterior vena cava both by the hepatic veins and also by the hepatic portal vein and its extensions via the renal portal system. The liver is thus situated on a venous "loop" whose ends have a common termination. Since veins enter this "loop"

at several sites, the blood they contribute may, in principle, flow into the posterior vena cava either along a venous pathway containing a valve (sphincter), or by passing through the liver. If the resistance to flow offered by the sphincter can change, and the venous inflow from other contributory veins varies, the venous return from any source may follow either of these routes on different occasions.

Akester (1964) has shown in anaesthetised fowls that radioactive material injected into the external iliac vein could travel by different routes depending on the state of the sphincter, between the extremes of passing directly to the posterior vena cava and being directed to the liver via the coccygeo-mesenteric vein.

There has been no quantitative measurement of liver blood flow in the turkey and only two workers have published results on liver blood flow in the fowl (Ranney, Chaikoff and Dobson, 1951; Sapirstein and Hartmann, 1959). Both these groups used radioactive material; Sapirstein and Hartman's indicator dilution method required also the death of the bird in order to digest the organs.



The present work was undertaken in an attempt to measure, by a simpler method, the total hepatic blood flow of unanaesthetised turkeys in the normal upright position, in such a way that the observations could be repeated at intervals when necessary. The present paper outlines the method. The results presented were obtained in normal birds, and in birds with ligatures placed either (a) on the posterior vena cava or (b) on the coecygeo-mesenteric vein at the venous arch (Fig. 1).

Bromsulphthalein (BSP) is a drug which is removed from the circulation almost entirely by the liver and excreted in the bile. There is no entero-hepatic circulation. It has been used as a test of liver function in man for many years. Richards, Tindall and Young (1959) showed, after a single injection in the dog, that the BSP content of the liver and the bile could be predicted from the fall in the plasma concentration. The fall in plasma concentration showed that a proportion (a) of the plasma content was accepted by the liver per unit time, and proportions (b and h) of the liver content were passed back into the plasma and into the bile per unit time, respectively. This relationship has been confirmed in the turkey (Clarkson 1961b). After a single injection the liver content rises rapidly to a peak and then falls in an exponential manner. At the peak of maximum content the liver is at input-output equilibrium with respect to blood and bile, i.e. the amount of BSP entering the liver from the plasma is exactly equal to the amount leaving the liver in the bile. At this point, and this point alone, the situation is directly comparable with that in continuous infusion taken to equilibrium. The time (t) of equilibrium can be calculated from the plasma curve, and the clearance of BSP at this time can be calculated. In practice there is no need to calculate t since it is readily shown that clearance at this point is a fraction  $\left(\frac{a \times h}{b+h}\right)$  of the plasma volume, V, per unit time.

Clearance is numerically equal to flow rate multiplied by the "extraction ratio" and a method has been devised for the separation of these two components of clearance. This will be published in detail elsewhere.

#### *Material and Methods*

*Turkeys.* Mature or nearly mature Beltsville White as well as a small number of Broad-breasted Bronze of either sex and weighing from 4.0 to 15.0 kg., were used.

*BSP.* This was given as a single injection of a sterile solution containing 50 mg./ml.\* For continuous infusion it was made up in a phosphate buffer of pH 7.30.

*Blood samples.* These were obtained from a catheter placed in the region of the heart via the jugular vein. Samples were taken at frequent

\* Savory & Moore Ltd, London

intervals and the plasma analysed for BSP using a Hilger & Watts Uvispek spectrophotometer, by a method similar to that previously described (Clarkson, 1961b).

Indocyanine green (ICG) was used in a few experiments.

*Operative procedure.* The birds were anaesthetised with halothane\* and oxygen and the appropriate vessel ligated. A single injection was given, the vessel ligated the next day and the test repeated 6-8 days later. The bird was then killed, the site of ligation inspected and the liver weighed and examined histologically.

### Experimental Results

#### Comparison of Clearance at Time $t$ and during Continuous Infusion

Three birds were given a single injection of BSP or ICG, followed by one or more periods of continuous infusion. Table 1 shows the clearances obtained.

TABLE 1

*Comparison of clearance obtained by continuous infusions and by single injection at  $t$  ...*

No.	Sex	Drug	$t$ min.	SINGLE INJECTION		CONTINUOUS INFUSION	
				Clearance at $t$ $\left( = \left( \frac{a \times h}{b+h} \right) V \right)$	Quantity removed at $t$ Conc <sup>a</sup> at $(t-0.5)$ min.	Clearance 1	Clearance
151	M	BSP	4.24	135.6	112.8	112.3	112.2
151	M	ICG	3.81	104.7	89.6	88.5	—
152	M	BSP	4.48	109.9	92.0	95.4	96.0
152	M	ICG	4.34	103.9	88.0	85.1	83.2
153	M	BSP	3.88	178.1	142.0	147.0	151.3
153	M	ICG	3.41	109.7	92.8	82.1	90.9

#### Ligation of Coccoygeo-mesenteric Vein

The results on 7 birds are shown in Table 2, giving a mean blood flow of 44.1 ml./kg./min. before ligation and a mean of 40.3 ml./kg./min. after ligation.

#### Ligation of Posterior Vena Cava

Table 3 shows the results in 9 birds, one of which was done twice (No. 158). In 3 of these birds the ligature had loosened and was not obstructing the flow of blood. Even if these birds are excluded the results are still variable, although 5 of the 7 show considerable increases including one (No. 35) in which the hepatic blood flow was almost doubled.

#### Liver Weights

Table 4 shows that the weight of the livers of birds whose posterior vena cava had been ligated was significantly increased over the controls and over those with a ligated coccoygeo-mesenteric vein.

\* "Fluothane" I.C.I.

TABLE 2

*The effect of ligation of the coccygeo-mesenteric vein on clearance, hepatic blood flow and extraction ratio of BSP*

No.	Sex	BEFORE LIGATION			AFTER LIGATION			Change in extraction in flow (per cent)	Change in extraction in flow (per cent)
		Clearance ml./kg./min.	Extraction per cent	Blood flow ml./kg./min.	Clearance ml./kg./min.	Extraction per cent	Blood flow ml./kg./min.		
81	M	17.2	54.1	31.8	15.2	44.0	34.6	-18.7	+8.8
3	F	13.9	41.5	33.5	13.4	43.3	30.9	+4.3	-7.8
12	M	16.5	46.5	35.4	16.8	36.4	46.0	-21.7	+29.9
115	F	20.1	44.7	44.9	18.5	42.4	43.7	-5.6	-2.7
52	F	20.8	44.5	46.7	13.8	46.0	30.0	+3.4	-35.8
24	M	29.0	51.0	57.0	18.6	37.2	50.0	-25.1	-12.3
6	M	31.1	52.3	59.4	17.9	42.5	42.0	-18.4	-29.3
Mean		21.1	47.8	44.1	16.8	41.7	40.3	-12.8	-8.6

TABLE 3

*The effect of ligation of the posterior vena cava on clearance, hepatic blood flow and extraction ratio of BSP*

No.	Sex	BEFORE LIGATION			AFTER LIGATION			Per cent change in extraction	Per cent change in blood flow
		Clearance ml./kg./min.	Extraction per cent	Blood flow ml./kg./min.	Clearance ml./kg./min.	Extraction per cent	Blood flow ml./kg./min.		
199	M	22.9	61.2	37.4	31.9	63.4	50.3	+3.5	+34.5
60	F	21.3	49.2	43.3	20.0	28.9	69.3	-41.3	+60.0
35	M	16.1	52.7	30.5	30.3	52.0	58.3	-1.3	+91.1
133	M	20.5	58.0	35.3	22.4	55.0	40.8	-5.2	+15.6
158	F	23.0	54.2	42.4	24.9	36.3	68.7*	-49.3	+62.0
69	M	32.5	61.1	53.2	18.1	53.3	34.0†	-1.7	-20.0
51	M	22.6	44.8	50.4	37.1	65.2	56.9	+6.7	+7.0
200	M	21.1	60.0	35.2	24.2	39.6	61.1†	-11.6	+21.2
45	M	19.2	44.7	43.0	17.7	57.5	30.8†	-4.2	-12.5
					24.7	62.0	39.8	+38.7	-7.4

\* One day after. † Ligature loose.

TABLE 4

*The effect of ligation of the coccygeo-mesenteric vein or the posterior vena cava on liver weight*

Procedure	No. of birds	Liver wt. g./kg. body wt.	
		Mean	Standard deviation
Normal	8	14.10	3.13
Ligation of posterior vena cava	10	20.07	3.84
Ligation of coccygeo-mesenteric vein	7	12.90	1.36

### *Discussion*

The basic description of the decremental graph of plasma concentration after single injection assumes that the changes produced in the plasma concentration by the action of the liver is evenly spaced throughout the whole plasma volume, mixing being virtually instantaneous, whether the substance introduced is the original injection or whether it is the continuous introduction of blood from the hepatic vein into the general circulatory "space".

It is possible to show that the events following single injection in the above conditions lead to the establishment of a "steady state" at the point of time at which liver content has its greatest value, and only at this time (*t*).

This steady state is thus the same as that existing in properly conducted continuous infusion work to determine clearance. Indeed, the purpose of continuous infusion is to continue indefinitely the brief period of steady state in single injection.

It would, therefore, be expected that a comparison of clearance determined from single injection and continuous infusion on the same animal, and in close succession, would show good agreement.

In practice the agreement is clear, but it is also found that clearances by single injection are greater than continuous infusion; the reasons for this are not yet known.

This is equivalent to a displacement in time of the plasma concentration relative to changes in the liver. It is reasonable to suppose that, in the turkey, the "time-displacement" is about 0.5 min. (cf. Table 1).

We have been unable to find any published figures of hepatic blood flow in the turkey with which to compare our results. Sapirstein and Hartman (1959) showed that the hepatic blood flow in 10 fowls was 38 ml./kg./min. In the 16 birds described in this paper the mean blood flow before any operative procedure was 44.2 ml./kg. min.

The interpretation of the effect of ligation of the coccygeo-mesenteric vein or the posterior vena cava is difficult due to the many variables

which could be involved. Our figures refer to the volume of blood passing along the hepatic veins, i.e. the total hepatic blood flow, and this can be influenced by alterations in any of the sources of the blood. For example, the flow from the splanchnic area could alter considerably, depending on the nervous state of the unanaesthetised bird. We are at present attempting to isolate this variable in birds with the coccygeo-mesenteric vein ligated using injections of adrenaline.

The results (Table 2) of the effect of ligation of the coccygeo-mesenteric vein could be explained by postulating that blood flow in either direction in this vein in the normal bird, depending on the state of the valve. If the valve was fully open blood may then flow from the liver to the kidneys and *vice versa* if the valve was fully closed. Since we have no knowledge of the state of the valve when the original injection was given, ligation could result in either a fall or an increase in liver blood flow. Since 5 of the 7 birds showed a fall in blood flow it is possible that normally a small amount of blood flows from the kidneys to the liver via the coccygeo-mesenteric vein and this was prevented from doing so by the ligature. However, we have little data at present on the variations in flow from day to day in an individual bird, and further experiments are needed before this suggestion can be confirmed.

Most of the birds with the posterior vena cava ligated show a considerable increase in flow. However, few of these are as high as might be expected if the whole of the renal blood and that from the hind limbs was directed through the liver. This is particularly true of Bird 45 (Table 3) where the blood flow after ligation was slightly reduced. This may have been due to collateral channels, possible diversion via the vertebral veins or caused by a considerable reduction in splanchnic flow which was sufficient to offset the increase in flow caused by the ligature.

These results are presented as a preliminary study of the problems posed by the complex venous connections of the avian liver, and to measure clearance, flow rate and extraction ratio.

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# 34

## AFFERENT PATHWAYS IN THE VAGUS AND THEIR INFLUENCE ON AVIAN BREATHING: A REVIEW

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### *Synopsis*

THE RESPIRATORY effects of bilateral cervical vagotomy in the bird are reviewed, including the varied changes in amplitude and rate, the characteristics of the slowing, the occurrence of dyspnoea, and the possible influence of anaesthesia, age, species and posture. The respiratory responses to stimulation of the central stump of the avian cervical vagus, and theoretical interpretations of the role of vagal afferent fibres in the control of breathing, are also briefly surveyed.

### *Introduction*

This review attempts to assemble the main essentials of what is known about the varying respiratory effects of bilateral cervical vagotomy in birds, and of the factors which may contribute to these variations. It also covers less fully the respiratory effects of stimulation of the central stump of the avian cervical vagus. Finally, theoretical interpretations of the role of vagal afferent pathways in the control of avian breathing are briefly outlined. It is hoped that such a review is warranted by the smallness of the space which the current reference books can afford for discussion of this important but unresolved physiological topic, and by the fact that the relevant papers, many of great length in French and German, are scattered over a century or more of literature.

### *Principal sources of literature*

The question of the afferent vagal control of breathing has recently been surveyed in reference books by Sturkie (1954) and Salt and Zeuthen (1960). Of the original papers the key works are those by Fedde, Burger and Kitchell (1963a, 1963b), and by Sinha (1958). These report extensive experiments and also provide a scholarly introduction to the literature. Those by Fedde *et al.* (1963a, b) are particularly helpful, for in addition to covering the literature before and

including the nineteenth century, they compare the effects of vagotomy in vertebrates generally, and re-examine the whole theoretical question of the afferent vagal control of breathing. Siefert's paper of 1896, an ambitious survey of reptilian and avian breathing, is unfortunately rather prolix, and his own original work is not systematically presented. Nevertheless, it provides an extensive and accurate analysis of the principal works of his day as well as comparisons with his own observations.

### *Bilateral cervical vagotomy*

The main points in dispute about the effects of bilateral cervical vagotomy in birds include the changes in the amplitude and rate of breathing, the nature of the slowing and the occurrence of dyspnoea. Among factors which may play a part in causing these variations are anaesthesia, age, species and posture of the animal.

### *Changes in Amplitude*

The amplitude of breathing has generally been found to increase greatly after bilateral vagotomy. This was certainly the conclusion reached by Cavalié (1898), Orr and Watson (1913), Saalfeld (1936), Graham (1940) and Fedde *et al.* (1963a). The descriptions of Zander (1879), Knoll (1880), Siefert (1896), Grober (1899) and Stübel (1910) also more or less definitely indicate an increase in amplitude; unfortunately many of the tracings in publications of this period have no preliminary records of normal breathing. Cavalié described the increase as considerable, and Saalfeld recorded an increase in the pigeon from a normal value of 5 ml. to an amplitude of 25 ml. after operation. Fedde *et al.* observed a 2- to 3-fold increase in conscious White Leghorn males; this increase developed rapidly during the first few minutes after section of the nerves, and appeared to be complete within 10 to 60 min. On the other hand, Couvreur (1892) regularly obtained a reduction in amplitude in the (presumably conscious) chicken and pigeon. Again Sinha (1958) reported a reduction in 64 out of 90 experiments on the anaesthetised or decerebrate pigeon, and in the rest of his experiments found no change in amplitude; he also noted a shift towards the inspiratory side.

remarked that in this species breathing slowed to a state of apnoea. Fedde *et al.* (1963a) closely studied the changes in rate in the male White Leghorn chicken. They found that the greatest change occurred during the first 5 min., but the maximum slowing was reached about 10 min. after operation. A transient initial stimulation of breathing attributable to mechanical stimulation of the nerve by cutting, was observed by Graham (1940) in the chicken, and by Sinha (1958) in the pigeon; it was also noted by Stübel (1910), but not in relation to any particular species.

A partial recovery of rate has been quite often observed. Boddaert (1862) described one experiment on a pigeon in which the rate became "pretty nearly normal" between the third and tenth day after operation. In the same species Zander (1879) found a considerable acceleration about 24 hr. after the nerves had been sectioned and Knoll (1880) and Siefert (1896) described a noticeable acceleration on the first or second day; Sinha (1958) observed "some recovery" within 6 to 12 hr. Couvreur (1892) found that in the chicken the rate accelerated during 4 to 5 days to about one-third of its normal value, while a somewhat greater acceleration occurred in the pigeon within 24 hr. Fedde *et al.* (1963a) obtained a partial recovery of rate in a few hours in older male chickens. Likewise, partial recovery was observed by Cavalié (1898) in the duck after a few days. Nevertheless, in all these reports the rate never recovered to its original value. Apparently only Eichhorst (1879) has claimed full recovery.

Not many workers have published tracings which enable the minute volume to be measured. However, the increase in amplitude observed by most investigators is certainly inadequate to compensate for the relatively enormous reduction in rate; therefore it may safely be assumed that the minute volume is profoundly depressed. Sinha (1958) directly demonstrated a fall in minute volume.

### *The Nature of the Slowing*

Pausing in the extreme expiratory position (Fig. 2) has been given by most authors as the explanation for the slowing of breathing after vagal section. This was observed in the pigeon by Knoll (1880), and confirmed by Grober (1899) and Grünwald (1904); Sinha (1958) reported its occurrence in the chicken. Siefert (1896), however, observed it only occasionally in his experiments on the pigeon. He believed that typically the pause coincided with the true resting position of the thorax, between the passive and active phases of inspiration: this he confirmed by showing that the position of the pause on his tracings was identical with that which he recorded after high transection of the spinal cord (Fig. 1). Sinha (1958) also observed arrest "in the middle position". On the other hand, a prolonged slow expiration (Fig. 5), without a pause at all, was found by Sinha (1958) in some of his experiments on the pigeon, and by Fedde *et al.* (1963a) in the chicken.

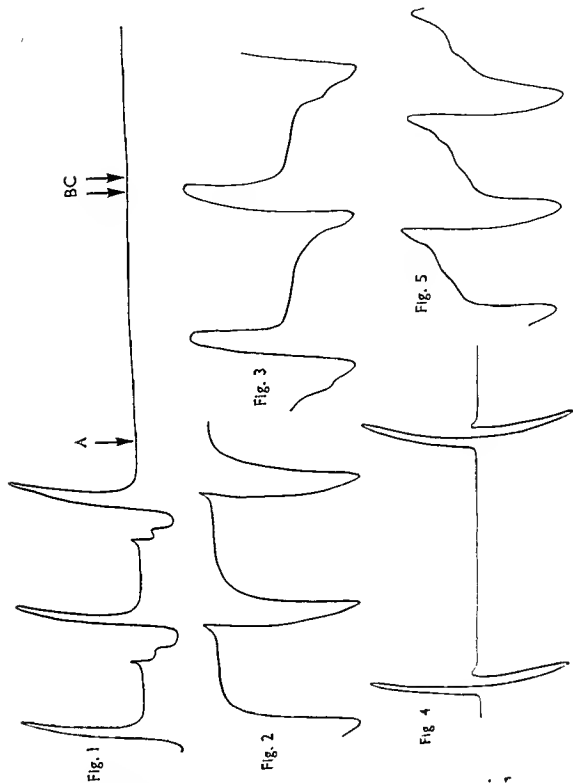


FIG. 1. Pauses in inspiration in the rest-inspiration of the thorax after bilateral cervical vagotomy in the pigeon. Point A indicates the onset and B the finish of Faradic stimulation of the central vagal stump. Point C indicates cutting the spinal cord high in the neck. From Siefert (1896). In this and the other figures inspiration is down and expiration up, as with a stethograph and tambour.

FIG. 2. Pauses at the end of expiration after bilateral cervical vagotomy in the pigeon. From Siefert (1896).

FIG. 3. Showing of inspiration after bilateral cervical vagotomy in the hooded crow. From Stübel (1910).

FIG. 4. Pauses in expiration after bilateral cervical vagotomy in the duck. From Stübel 1910.

FIG. 5. Showing of expiration after bilateral cervical vagotomy in a species of crow. From Stübel 1910.

By far the most complete survey of the pattern of breathing after cervical vagotomy was given by Stübel (1910). After experiments on several wild and domestic avian species he strongly confirmed Siefert's (1896) conclusion that pauses in the resting position of the thorax are far more common than pauses at the end of expiration. However, he also showed that these pauses in the resting position can interrupt either expiration (Fig. 4) or inspiration (Fig. 1). Between the pauses the individual breaths tend to be faster than normal. The slowing of breathing can also arise from slowing of either inspiration (Fig. 3) or expiration (Fig. 5); this seemed to occur relatively commonly in predatory birds and in ravens and crows. In any species short and erratic breathing movements may be irregularly interspersed between the deeper breaths.

### *Dyspnoea*

In a detailed account of two of his many experiments on pigeons Boddaert (1862) mentioned severe dyspnoea resulting from vagotomy. Zander (1879) always obtained dyspnoea in the pigeon, but it was not invariably severe. It was often observed by Grober (1899) in the young pigeon, but in the same species Sinha (1958) saw no signs of asphyxia. Orr and Watson (1913) reported asphyxial struggles in the duck. In the chicken Fedde *et al.* (1963a) found that conscious male birds 32 weeks of age generally died within 3 hr. with symptoms resembling those of asphyxia but this did not happen when the birds were only 16 weeks old. It would evidently appear that Boddaert (1862) usually managed to keep his pigeons alive for 8 to 12 days, until they died of starvation, whereas Couvreur (1892) and Grober (1899) implied that their birds usually died within a few days. On the other hand, Stübel (1910) apparently believed that dyspnoea could be avoided by using appropriate techniques for recording breathing. Other authors (e.g. Siefert, 1896) made no mention of dyspnoea, or of fatal termination, in their own experiments.

### *Anaesthesia*

As Fedde *et al.* (1963a) pointed out, the introduction of anaesthesia into physiology towards the end of the nineteenth century could be one reason for the varied results of vagotomy. Most workers of this period (e.g. Couvreur, 1892; Siefert, 1896; Cavalié, 1898; Grober, 1899) gave no systematic account of their experimental methods so that it is impossible to decide whether their animals were anaesthetised or not. Saalfeld (1936) used intraperitoneal urethane and ether, and Graham (1940) used barbiturates. A variety of non-volatile anaesthetics were used by Sinha (1958), as well as decerebration; he reported no correlation of response with any particular type of anaesthetic. Fedde *et al.* (1963a), on the other hand, showed that responses to bilateral vagotomy are different in the conscious male chicken as compared with similar male birds anaesthetised by intravenous sodium pentobarbital.

The effect of anaesthesia was to restore the rate and amplitude towards normal values. The extent of this recovery was proportional to the depth of anaesthesia; the deeper the anaesthesia the more nearly normal the breathing became. Nevertheless, as a general rule, anaesthesia seemed unable to prevent entirely some characteristic slowing of rate and increase of amplitude. As will be mentioned later, this discovery of the effect of barbiturate anaesthesia has interesting theoretical repercussions.

### *Age*

Most workers (e.g. Couvreur, 1892; Sicfert, 1896; Cavalié, 1898; Orr and Watson, 1913) omitted all mention of the age of their experimental animals, but Grober (1899) noted that "young" pigeons often died. Saalfeld (1936) and Sinha (1958) clearly stated that they used adult pigeons. Fedde *et al.* (1963a) obtained in older male chickens of 32 weeks a progressive decline in amplitude and rise in rate, culminating in death within 3 hr.; male birds of 16 weeks maintained a stable amplitude and rate during the same period.

### *Species*

Very little is known about the influence of species and breed on the results of bilateral vagotomy. Fedde *et al.* (1963a) asserted that genetic differences constitute a source of variation, and that heavy breeds of fowl typically show a somewhat prolonged inspiration and less intense pulmonary changes. These investigators also attributed to Stübel (1910) the view that species differences should be considered a large factor. In fact, Stübel seems to have been more impressed with the considerable variations which he observed in individuals of the same and different species, both in normal breathing and after vagotomy, and remarked that "it is impossible to establish characteristic differences in the respiratory curves of the various avian species". He did conclude, however, that the greatest slowing after vagotomy occurs in the duck, with pauses of up to 1 min. Next to this he thought the hen most severely affected. Having made observations on crows, ravens and several predators he also tentatively suggested that the impairment of breathing after bilateral vagotomy appears to be less severe in strong-flying birds than in the inert domestic species. He emphasised, however, that since his material was not abundant, too much weight could not be placed on this conclusion.

their methods, but one may assume that most of them placed the animal in the supine posture for ease of restraint. Only Saalfeld (1936) appears to have used both the supine and the prone posture: he observed no effect on the result. In view of the various influences that can affect breathing in the entirely abnormal supine posture (see Salt and Zeuthen, 1960; King and Payne, 1964), the possibility that it may modify the effects of vagotomy cannot be safely disregarded.

*Respiratory effects of stimulating the central stump of the vagus*

The effects of electrical stimulation of the central stump of the cut cervical vagus are variable. As in mammals the response depends on the frequency, etc., of the stimulus. Siefert (1896) and Grober (1899) summed up the already abundant experimental evidence of their day, gained mainly from pigeons, with the generalisation that stimulation only rarely produced arrest in the deep inspiratory position; usually it produced arrest in the expiratory position, or in the position as the arrest which characterised bilateral vagotomy, i.e. in the resting position of the thorax (Fig. 1). In the chicken, however, Graham (1940) found a tendency for a shift to the inspiratory phase, with a general slowing of breathing. Slowing was also invariably obtained by Dooley and Koppányi (1929) in the duck, regardless of the "strength" of the current; this they regarded as contrary to the usual effect in mammals. On the other hand, in the same species Cavalié (1898) obtained acceleration of breathing with weak stimulation, and arrest in the expiratory phase with strong stimulation. Orr and Watson (1913) usually obtained single inspiratory movements in the duck, synchronous with stimulation.

Sinha (1958) thoroughly reviewed the whole field and greatly extended knowledge by using properly controlled frequencies and voltages. Intense stimuli at low frequency induced a shift towards inspiration with increased rate. Weak stimuli at higher frequency induced a slight expiratory reaction with slowing or acceleration of breathing. Stronger stimuli at higher frequencies induced an inspiratory tonic component. He concluded that these results are similar to those obtained from mammals.

*Interpretation of effects of bilateral cervical vagotomy*

The effects of bilateral cervical vagotomy are different in birds and mammals. The responses in birds, although erratic enough in detail, are apparently relatively consistent in their basic characteristics: in mammals the nature and severity of the changes have evidently varied considerably with the observer (Bozler and Bureh, 1951). In birds the enormous reduction in rate is due most commonly to a pause, occurring in the resting position of the thorax (i.e. midway between the active and passive phases of inspiration and/or expiration); it may also be due to a general slowing of either inspiration or expiration. In



the mammal, however, the slowing is much smaller and may often be due to a prolongation of inspiration. In birds the full effects on amplitude and rate develop progressively over 10 min. or more; in mammals they may be immediate. The minute volume is profoundly depressed in birds, but may be only slightly altered in mammals (Best and Taylor, 1955). Dyspnoea seems to be more common in the bird than in the mammal.

The usual interpretation of the effects of bilateral cervical vagotomy in birds is that breathing is controlled by Hering-Breuer reflexes (see Sturkie, 1954; Salt and Zeuthen, 1960); the slow rate of breathing after bilateral vagotomy is held to reflect the spontaneous activity of the respiratory centre (Salt and Zeuthen, 1960). Fedde *et al.* (1963a, b) have strongly challenged this view. They consider the hypothesis that stimulatory afferent vagal impulses normally excite the respiratory centres: these arise from unknown receptors and are unaffected by changes in the tension of the lung. (This was considered by Bozler and Burch (1951), from their experiments on dogs.) Thus, it is the absence of these impulses which causes the slowness of breathing after vagotomy. The fact that appropriate electrical stimulation of the central stump of the avian vagus can cause faster breathing appears to support the concept of a stimulatory drive in the avian vagus. Fedde *et al.* (1963a, b) also point out, however, that many other afferent impulses must reach the avian respiratory centre, such as from receptors of CO<sub>2</sub>, pain and temperature. These they postulate as inhibitory to the centre. Hence they interpret the large decrease in the rate of breathing after vagotomy as "the full expression of these inhibitory impulses": this is "normally counter-balanced by vagal stimulatory impulses". Deep anaesthesia by appropriate drugs, after bilateral cervical vagotomy, could diminish the responsiveness of the centre to these inhibitory impulses. Their own important experimental results of vagotomy under deep barbiturate anaesthesia can then be interpreted as arising from the blocking of both the stimulatory and the inhibitory afferent impulses. According to this proposition the pattern of breathing observed after vagotomy under deep anaesthesia must then be the expression of the basic activity of the respiratory centre; this pattern is observed to resemble quite closely that of normal breathing, the only difference being a small slowing of the rate and a small increase in the amplitude of breathing.

inhibitory non-vagal impulses? These problems await further investigation.

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# INDEX OF NAMES

*Entries in italics refer to papers in this volume. References made by authors to their own work are not indexed separately.*

- Adkins, J. S., 164, 170  
Ainsworth, L., 23, 29  
Aitken, R. N. C., 33, 34, 38  
Akester, A. R., 289-92, 295, 300  
Albada, M. van, 212, 226  
Allen, T. E., 41, 43  
Almquist, H. J., 204, 207  
Alsborne, K., 53, 60  
Altzuler, N., 120, 122  
Annison, E. F., 184, 188  
Ariyoshi, S., 147, 148, 153  
Armstrong, D. I., 120, 122  
Ashoub, M. B., 230, 234  
Atkinson, J. C., 230, 234  
Azari, P. R., 131, 132; 140, 144
- Back, N., 119, 123  
Badman, H. G., 114, 123, 135, 144  
Badreldin, A. L., 56, 59  
Baghdiantz, A., 201, 202  
Bailey, G. E., 76, 86  
Baillie, A. H., 32-38  
Bajapi, P. K., 56, 59  
Baker, Ann C. M., 138-43  
Bandemer, S. L., 136, 144  
Bandi, L., 36, 38  
Barott, H. G., 212, 226, 229-34  
Bauernfeind, J. C., 77-86  
Baum, G. J., 111, 112  
Bautzmann, H., 275, 278  
Bayley, H. S., 16, 21, 75-86  
Beadle, B. W., 197, 198  
Beane, W. L., 104, 109-112  
Beattie, J., 228, 234  
Becker, Y., 131, 132  
Bell, D. J., 50, 104, 111, 114, 122  
Bennett, M. A., 87, 101  
Bennett, N., 138, 144  
Bernstein, S., 100, 102  
Bert, H. M., 148, 153  
Berthet, J., 122, 123  
Best, C. H., 309, 310  
Bethune, J. E., 119, 123  
Bickoff, E. M., 76, 86  
Biely, J., 168, 170  
Bierman, E. L., 120, 123  
Biggers, J. D., 230, 234  
Bird, S., 230, 234  
Bishop, J. S., 120, 122
- Blaber, L. C., 252-9, 264, 272, 276, 278  
Black, D. J., 75, 86  
Blyth, J. S. S., 64, 74  
Boas, N. F., 104, 110, 112  
Bobr, L. W., 39-43  
Boddaert, R., 304, 306, 310  
Bodo, R. C. De, cf. De Bodo, R. C.  
Boersma, H. Y., 204, 208  
Bolton, W., 134, 142, 143  
Bonadonna, T., 55, 59  
Bonting, S. L., 276, 278  
Borchert, K. L., 212, 226  
Bose, S., 147, 150, 153  
Bottino, N. R., 100, 101  
Bowman, W. C., 249-59, 261-72  
Boyle, G. J., 105, 112  
Bozler, E., 308, 309, 310  
Brandt, J. W. A., 134-44  
Bray, D. J., 168, 170, 237, 241  
Brin, M., 90, 102  
Brodie, B. B., 120, 123  
Brody, S., 229, 234  
Brooks, J., 72, 74, 204, 207  
Brown, D. J., 104, 111  
Brown, G. L., 249, 259  
Brown, J. B., 24-9  
Brown, J. G., 73, 74  
Brown, K. I., 104, 111  
Brown, P. S., 21, 22  
Brown, W. O., 128, 132, 133-45, 150-3  
Brownlee, G., 268, 272  
Brubacher, G., 77, 85, 86  
Bruckner-Kardoss, E., 182, 183  
Buchanan, J. H., 88, 101  
Budowski, P., 100, 101  
Bulbrang, E., 269, 272  
Bunnell, R. H., 81, 83, 86  
Burak, W. A., 119, 123  
Burch, B. H., 302-10  
Burger, R. E., 302-10  
Burmester, B. R., 11, 13, 204, 212  
Burn, J. H., 261, 273  
Bush, H., 131, 132
- Callingham, B. A., 240, 250, 255  
Calman, 32, 37  
Calman, H. I., 36-8  
Cameron, I. C., 201, 202  
Cameron, L. H. D., 37, 38



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# INDEX OF NAMES

*Entries in italics refer to papers in this volume. References made by authors to their own work are not indexed separately.*

- Adkins, J. S., 164, 170  
Ainsworth, L., 23, 29  
Aitken, R. N. C., 33, 34, 38  
Akester, A. R., 289-92, 295, 300  
Albada, M. van, 212, 226  
Allen, T. E., 41, 43  
Almquist, H. J., 204, 207  
Alsborne, K., 53, 60  
Altzuler, N., 120, 122  
Annison, E. F., 184, 188  
Ariyoshi, S., 147, 148, 153  
Armstrong, D. I., 120, 122  
Ashoub, M. B., 230, 234  
Atkinson, J. C., 230, 234  
Azari, P. R., 131, 132; 140, 144
- Back, N., 119, 123  
Badman, H. G., 114, 123, 135, 144  
Badreldin, A. L., 56, 59  
Baghdiantz, A., 201, 202  
Bailey, G. E., 76, 86  
Baillie, A. H., 32-38  
Bajapi, P. K., 56, 59  
Baker, Ann C. M., 138-43  
Bandemer, S. L., 136, 144  
Bandi, L., 36, 38  
Barott, H. G., 212, 226, 229-34  
Bauernfeind, J. C., 77-86  
Baum, G. J., 111, 112  
Bautzmann, H., 275, 278  
Bayley, H. S., 16, 21, 75-86  
Beadle, B. W., 197, 198  
Beane, W. L., 104, 109-112
- Blaber, L. C., 252-9, 264, 272, 276, 278  
Black, D. J., 75, 86  
Blyth, J. S. S., 64, 74  
Boas, N. F., 104, 110, 112  
Bobr, L. W., 39-43  
Boddaert, R., 304, 306, 310  
Bodo, R. C. De, *cf.* De Bodo, R. C.  
Boersma, H. Y., 204, 208  
Bolton, W., 134, 142, 143  
Bonadonna, T., 55, 59  
Bonting, S. L., 276, 278  
Borchert, K. L., 212, 226  
Bose, S., 147, 150, 153  
Bottino, N. R., 100, 101  
Bowman, W. C., 249-59, 261-72  
Boyle, G. J., 105, 112  
Bozler, E., 308, 309, 310  
Brandt, J. W. A., 134-44  
Bray, D. J., 168, 170, 237, 241  
Brin, M., 90, 102  
Brodie, B. B., 120, 123  
Brody, S., 229, 234  
Brooks, J., 72, 74, 204, 207  
Brown, D. J., 104, 111  
Brown, G. L., 249, 259  
Brown, J. B., 24-9  
Brown, J. G., 73, 74  
Brown, K. I., 104, 111  
Brown, P. S., 21, 22  
Brown, W. O., 128, 132, 133-45, 150-3  
Brownlee, G., 268, 272  
Brubacher, G., 77, 85, 86  
Bruckner-Kardos, E., 182, 183  
Buchanan, J. H., 88, 101  
Budowski, P., 100, 101  
Buller, E., 269, 272 ..

- Campbell, R. C., 178, 180  
 Cantoni, G. L., 88, 101  
 Carayon-Gentil, A., 258, 260  
 Carey, N. H., 125-32, 135, 144  
 Carlson, L. A., 120-3  
 Caro, L. G., 131, 132  
 Carpenter, K. J., 171, 180  
 Carpenter, T. M., 214, 226  
 Carver, J. S., 147, 154  
 Cass, Rosemary, 279-85  
 Cavalié, M., 303-10  
 Cayer, D., 88, 101  
 Chabrier, P., 258, 260  
 Chaikoff, I. L., 35, 37, 88, 101, 295, 300  
 Challey, J. R., 110, 111  
 Chance, M. R. A., 230, 234  
 Chang, T. S., 103, 111  
 Chapman, A. B., 53, 59  
 Charles, D. R., 238, 241  
 Cheney, B. A., 201, 202  
 Cheymol, J., 258, 260  
 Clarke, M. H., 16, 21  
 Clarkson, M. J., 291, 293, 294-300  
 Clements, L. P., 275, 278  
 Coates, M. E., 88, 101, 181-88  
 Cohen, J. A., 257, 259  
 Cohn, G. L., 35, 38  
 Combs, G. F., 163, 170  
 Common, R. H., 23, 29, 134, 143, 144  
 Connally, J. D., 110, 112  
 Conner, M. H., 109, 111  
 Conrad, D. H., 235, 236, 241  
 Conrad, R. M., 197, 198  
 Constable, B. J., 104, 110, 112  
 Cooper, D. M., 53, 59  
 Copp, D. H., 201, 202  
 Corkill, A. B., 3, 9  
 Cornatzer, W. E., 88, 101  
 Cosida, E., 53, 59  
 Cotterill, O., 140, 144  
 Couch, J. R., 87, 99, 101  
 Coulson, E. J., 146, 153  
 Court, S. A., 143, 144  
 Couvreur, E., 303-10  
 Craig, J. V., 53, 59  
 Crema, A., 269, 272  
 Cuthbert, A. W., 255-9, 274-8  
  
 Daggy, E. E., 76, 86  
 Dalton, A. J., 131, 132  
 Darnell, J. E., 131, 132  
 Davidson, A. C. F., 201, 202  
 Davies, R. E., 99, 101  
 Day, M., 268, 273  
 Dean, W. F., 173, 180  
 Deane, H. W., 35, 37, 38  
 De Bodo, R. C., 120, 122  
 Debos, A. F., 120, 123  
 de Duve, C., *cf.* Duve, C. de  
 Deeb, S. S., 57, 60  
  
 De Ritter, E., 77, 86  
 Desmarais, A., 58, 59  
 De Somer, P., 186, 187  
 Despointes, R. H., 119, 123  
 Deuel, H. J., 88, 101  
 Deuel, H. J. Jnr, 76-86  
 Deutsch, N. M., 104, 112  
 Diezsfalusy, E., 26, 29  
 Dixon, J. M., 147, 153  
 Dobson, E. L., 295, 300  
 Doehl, R., 53, 59  
 Dole, V. P., 113-23  
 Donovan, G. A., 230, 234  
 Dooley, M., 308, 310  
 Doran, B. M., 89-101  
 Dougherty, J. E., 212, 227, 231-4  
 Drieling, D. A., 120, 123  
 Driks, E. C., 35, 38  
 Drimmelen, G. C. Van, *cf.* Van Drimmelen, G. C.  
 Drucker, W. D., 36, 38  
 Ducay, E. D., 134-44  
 Dunn, A., 120, 122  
 Durrum, E. L., 136, 144  
 Duve, C. de, 122, 124  
  
 Eckstein, B., 53, 59, 104-12  
 Eckstein, H. C., 88, 101  
 Eichhorst, H., 304, 310  
 Eilat, A., 103-12  
 Ekman, P., 146, 153, 214, 226  
 Elford, H. L., 88, 101  
 El Jack, M. H., 44-51  
 Elsbach, P., 120, 123  
 Elton, R. L., 104-10  
 Emanuelson, H., 146, 153  
 Engel, L. L., 35, 38  
 Erickson, B. N., 100, 102  
 Erspamer, V., 267, 273  
 Escher, H. H., 75, 86  
 Eshkol, Z., 104, 112  
 Esmay, M. L., 212, 226  
 Euler, U. S. von, 284, 285  
 Evans, D. H. L., 275, 278  
 Evans, E. M., 275, 278  
 Evans, R. J., 136, 144  
 Everett, S. D., 261-73, 277, 278  
 Ewald, W., 35, 37  
 Ewing, W. R., 76, 86  
 Eyssen, H., 186, 187  
  
 Farr, A. C., 138, 144  
 Featherstone, R. M., 276, 278  
 Fedde, M. R., 302-10  
 Feeney, R. E., 131, 132, 138-45  
 Feigelson, E. B., 119, 123  
 Feldberg, W., 279, 285  
 Ferguson, J., 275, 278  
 Ferguson, M. M., 32-38

- Ferguson, T. M., 87, 96-101  
 Fields, J., 146, 153  
 Fisher, H., 148, 153, 156, 162, 164-70  
 Florentino, R. F., 158, 162  
 Forbes, M., 187, 188  
 Forchielli, E., 35, 38  
 Ford, A. G., 146, 153  
 Foster, G. V., 201, 202  
 Fransonn, A., 146, 153  
 Fraps, R. M., 5-10, 12, 13, 23, 29  
 Freedman, S. L., 193, 198  
 Freeman, B. M., 103-11  
 Fritz, J. C., 212, 226  
 Fry, E. G., 104, 112  
 Fujishima, T., 47, 51  
 Fulton, M. P., 257, 259  
 Furman, M., 56, 59  
 Furuta, F., 183, 188  
 Fussell, M. H., 147, 153  
  
 Gandia, H., 289, 293  
 Ganguly, J., 77, 86  
 Garcia, T. P., 57, 59  
 Gauthier, G. F., 201, 202  
 Geake, 204  
 Gentil, A. Carayon-, *cf.* Carayon-Gentil, A.  
 Gerriets, E., 212, 226  
 Gessell, J. A., 237, 241  
 Giebisch, G., 72-4  
 Gilbert, A. B., 11-13, 289, 293  
 Gillam, A. E., 75, 77, 86  
 Ginsborg, B. L., 249, 259  
 Glenner, G. G., 131, 132  
 Glick, B., 103-12  
 Gloor, V., 77, 86  
 Goldberg, A. A. J., 249, 259  
 Goldfien, A., 119, 123  
 Good, C. A., 105, 111  
 Goodwin, T. W., 76-8, 86  
 Gordon, H. A., 182, 188  
 Graham, J. D. P., 303-10  
 Greenberg, D. M., 88, 101  
 Gregory, M. E., 184, 187  
 Griffiths, K., 35-7  
 Grigg, G. W., 40, 43  
 Grimbleby, F. H., 75, 86  
 Grober, J. A., 303-10  
 Grose, F., 32, 38  
 Grünwald, J., 304, 310  
 Gush, D. G. M. Wood-, *cf.* Wood-Gush,  
     D. G. M.  
 Gutteridge, H. S., 231, 234  
 Guttridge, D. G. A., 178, 180  
  
 Hallmann, L. F., 88, 101  
 Halpern, P. E., 87, 101  
 Hamilton, J. A., 35, 38  
 Hamilton, R. E., 88, 101  
 Harper, A. E., 156, 162, 164, 170  
 Harrison, G. F., 88, 101, 185, 187  
 Hart, D. McK., 32-7  
 Hartmann, F., 87, 88, 101  
 Hartman, F. A., 295, 299, 301  
 Harvey, A. M., 249, 259  
 Harvey, C. N., 242, 245  
 Haslewood, G. A. D., 105, 111  
 Hazaard, R., 258, 260  
 Hazelwood, R. L., 115, 123  
 Heald, P. J., 113-23  
 Hecht, L. I., 129, 132  
 Heilbron, L. M., 75, 77, 86  
 Hendler, R. W., 126, 130-2  
 Heneghan, J. B., 182, 188  
 Henze, K. G., 201, 202  
 Hershey, J. M., 88, 101  
 Hertelendy, F., 201, 202  
 Hertz, R., 134, 142, 144  
 Hevesy, G., 100, 101  
 Hill, K. J., 184, 188  
 Hill, R. T., 3, 9  
 Hill, S. R., 212, 226  
 Hirsch, P. F., 201, 202  
 Hizikuro, S., 148, 153  
 Hoagland, M. B., 129, 132  
 Hodges, R. D., 191-8  
 Hokfelt, B., 35, 38  
 Holzbauer, M., 281, 285  
 Hoover, G. N., 73, 74, 141, 145  
 Hoppe, J. O., 252, 260  
 Horne, R. W., 131, 132  
 Horning, M. G., 88, 101  
 Howard, A. N., 104, 110, 112  
 Hsu, K. C., 131, 132  
 Hugget, A. St. G., 115, 123  
 Huggins, C., 49, 50  
 Hughes, J. S., 77, 86  
 Hughes, J. S., 146, 153  
 Huhtanen, C. N., 186, 188  
 Hummel, E. C., 100, 102  
 Hunsaker, W. G., 197, 198  
 Hunton, P., 16, 21  
 Huston, T. M., 7-13  
 Hutchinson, J. C. D., 242, 245  
  
 Honen, M., 35-38  
 Isacks, R. E., 90, 101  
  
 Jaap, R. G., 103, 111  
 Jack, M. H. *cf.* El Jack, M. H.  
 Jackson, N., 131, 144  
 Jailer, J. W., 104, 110, 112  
 Jayne-Williams, D. J., *cf.* 2

- Jeffery, A. M., 147, 153  
 Jin Soon Ju, *cf.* Ju, J. S.  
 John, D. L. St., *cf.* St. John, D. L.  
 Johnson, D., 148, 153, 164, 168, 170  
 Johnson, E. S., 268-73  
 Johnson, O., 147, 154  
 Johnston, H. S., 32, 34, 38  
 Jones, M., 276, 278  
 Joralemon, J., 87, 101  
 Ju, J. S., 148, 153  
 Jucker, E., 75, 86  
  
 Kadin, H., 77, 86  
 Kamar, G. A. R., 56, 59  
 Kardoss, E. Bruckner-, *cf.* Bruckner-  
     Kardoss, E.  
 Karmen, A., 119, 123  
 Karrer, P., 75, 86  
 Katayama, T., 147, 153  
 Kelley, M. A. R., 212, 227  
 Kempster, H. L., 77, 86  
 Kennedy, G. H., 80, 83, 86  
 Key, B. J., 279, 285  
 King, A. S., 302-10  
 King, J. R., 214, 226  
 Kitchell, R. L., 302-10  
 Kleiber, M., 211-27, 231-4  
 Klymiukowna, B., 50  
 Knight, C. A., 140, 145  
 Knobloch, E., 105, 112  
 Knoblock, E., 119, 123  
 Knoll, P., 303, 304, 310  
 Koelle, G. B., 275, 276, 278  
 Koike, T., 183, 188  
 Kon, S. K., 88, 101  
 Koppányi, T., 308, 310  
 Korner, A., 129, 132  
 Kosin, I. L., 42, 43  
 Kramer, H., 105, 111  
 Krey, H. P. Van, *cf.* Van Krey, H. P.  
 Kronfeld, D. S., 114, 122, 123  
 Krop, S., 257, 260  
 Kruhffer, P., 64, 74  
 Krüskemper, H. L., 35, 38  
 Krzanowska, H., 41, 43  
 Kubota, D., 147-53  
 Kuether, C. A., 105, 112  
 Kuhn, R., 75, 86  
 Kuljian, A., 35, 38  
 Kumar, M. A., 201, 202  
 Kumaran, J. D. S., 53, 59  
 Kuo, Z. Y., 275, 278  
  
 Lacassagne, L., 195, 198  
 Laerdal, Osm A. M., 147, 150, 153  
 Lake, P. E., 11-13, 40-3, 44-51, 53, 59  
 Lamming, G. L., 238, 241  
 Lampman, C. E., 235, 236, 241  
 Laurell, S., 120, 123  
  
 Layne, D. S., 23, 28, 29  
 Lederer, E., 75, 86  
 Leibson, L. G., 283-5  
 Lepkovsky, S., 183, 188  
 Lerner, I., 231, 234  
 Lev, M., 187, 188  
 Levi, H. B., 100, 101  
 Levy, H., 35, 38  
 Lewis, D., 155-62, 163-70, 171-80, 238, 241  
 Lewis, H. L., 104, 112  
 Lewis, P. R., 275-8  
 Liang, T.-Y., 104, 112  
 Lieberman, S., 36-8  
 Liljedahl, S. O., 120, 123  
 Lin, R. C. Y., 269, 272  
 Lincoln, D., 239, 241  
 Linnane, A. W., 131, 132  
 Lipsett, M. B., 35, 38  
 Lisboa, B. P., 26, 29  
 Livingston, A. L., 76, 86  
 Lobel, B. L., 35, 37  
 Lokhorst, W., 211-27  
 Long, C. N. H., 104, 112  
 Lorenz, F. W., 39-43, 115, 123  
 Loughlin, R. E., 88, 101  
 Lowry, O. H., 138, 144  
 Lush, I. E., 138, 140, 144  
 Lutzenberg, F., 53, 59  
  
 McDaniel, G. R., 53, 59  
 MacDonald, A. J., 147, 150, 153  
 MacDonnell, L. R., 140, 144, 145  
 McDougall, B. M., 88, 101  
 MacIntyre, I., 201, 202  
 McLaren, A., 230, 234  
 Macrae, H. F., 23, 29  
 McSpadden, B. J., 55, 59  
 Macy, I. G., 100, 102  
 Magsood, M., 59  
 Maickel, R. P., 120, 123  
 Mandels, S., 134-44  
 Mann, T., 44, 49, 51  
 Manwell, C., 138-43  
 March, B. E., 168, 170, 178, 180  
 Marley, E., 279, 285  
 Martin, E. W., 143, 144  
 Marusich, W. Y., 77-86  
 Masoro, E. J., 114, 122, 123  
 Mattson, F. H., 76, 83, 86  
 Maw, M. A., 23, 29  
 Mehl, J. W., 77, 86  
 Mehlman, B., 119, 123  
 Meinertz, H., 115, 123  
 Meyer, J. H., 148, 153, 154  
 Meyer, R. K., 103-12  
 Miahle, P., 119, 123  
 Michel, H. O., 257, 260  
 Michie, D., 230, 234  
 Milner, C. K., 178, 180  
 Mitchell, H. H., 148, 153, 212, 227

- Mitchell, Margaret E., 14-22  
 Moge, G. A., 257, 259  
 Mongin, P., 195, 198  
 Moore, D., 207, 208  
 Moore, J. H., 87-101, 185, 187  
 Moore, S. A., 147, 154  
 Morgan, C., 131, 132  
 Morimoto, H., 147, 148, 153  
 Morris, T. R., 15, 17, 22, 201, 202  
 Morton, R. K., 131, 132  
 Mount, L. E., 229, 234  
 Mueller, A. P., 103, 105, 112  
 Mueller, W. J., 235-41  
 Mulrow, P. H., 35, 38  
 Munson, P. L., 201, 202
- Nagington, J., 131, 132  
 Nagra, C. L., 111, 112  
 Nakajo, S., 12, 13  
 Nalbandov, A. V., vi, 3-10, 11-22, 134-7,  
 143-4  
 Nasset, E. S., 148, 153  
 National Research Council, 79, 86, 170,  
 173, 180, 237, 241  
 Needham, J., 99, 101  
 Nelson, D. M., 3-9  
 Newberne, P. M., 147, 153  
 Newcomer, W. S., 104, 110, 112  
 Newlands, Gillian M., 75-86  
 Niemi, M., 35-38  
 Nishiyama, H., 46, 47, 51
- Palmer, L. S., 77, 86  
 Parades, J. R., 57, 59  
 Parker, J. E., 55, 59  
 Parks, A. S., vi, 3, 9  
 Parlow, A., 4, 10  
 Paoletti, R., 120, 123  
 Paton, W. M. D., 262, 266, 273  
 Payne, C. G., 156, 162, 163-70, 235-41,  
 242-5  
 Payne, D. C., 308, 310  
 Payne, L. F., 77, 86  
 Pearson, B., 32, 38  
 Pearson, W. E., 80, 86  
 Pearson, W. N., 158, 162  
 Penman, S., 131, 132  
 Pensack, J. M., 186, 188  
 Perck, M., 52-60, 103-12  
 Perlmann, G. E., 143, 144  
 Perlman, I., 88, 101  
 Pero, R., 212, 227  
 Pesonen, S., 35, 38  
 Peterson, C. F., 235, 236, 241  
 Peterson, W. J., 77, 80, 86  
 Pfaff, W. W., 119, 123  
 Pilgeram, L. O., 88, 101  
 Polge, C., 56, 59  
 Polskin, L. J., 84, 86  
 Porter, J. W. G., 184, 187  
 Poulik, M. D., 138, 144  
 Pozzi, G. G., 55, 59  
 Pytasz, M., 50  
 Pringle, E. M., 212, 226, 229-34

- Romijn, C., vi, 211-27  
 Romney, S. L., 35, 37  
 Rookledge, K. A., 114, 115, 122, 123  
 Rosenbrough, N. J., 138, 144  
 Rosenthal, S., 88, 101  
 Rothchild, J., 5-13  
 Roy, E. J., 24, 29  
 Rozin, S., 49, 51  
 Rubin, B. L., 35, 38  
 Russell, W. C., 84, 86  
 Rutledge, W. A., 134, 143, 144  
 Ryan, R. K., 53, 59
- Saalfeld, E. von, 303-10  
 Saeki, Y., 56, 59  
 Saharia, W., 23, 29  
 Sainsbury, D. W. B., 212, 227, 229, 234, 242-5  
 Salmon, W. D., 155, 162  
 Salt, G. W., 302-10  
 Sang, J. H., 64, 74  
 Sanker, D. V. Siva, *cf.* Siva Sanker, D. V.  
 Sapirstein, L. A., 295, 299, 301  
 Sarcione, E. J., 119, 123  
 Sato, K., 104, 110, 112  
 Sauter, E. A., 235, 236, 241  
 Savage, J. E., 147, 153  
 Sayers, G., 104, 112  
 Sayers, M. A., 104, 112  
 Schjeide, O. A., 99, 101  
 Schnell, R. B., 230, 234  
 Schoorl, P., 204, 208  
 Schroder, R., 275, 278  
 Schueler, F. W., 266, 273  
 Schuman, H. J., 119, 123  
 Schwartz, I. L., 120, 123  
 Scott, H. M., 138, 145, 173, 180, 197, 198  
 Scott, J. F., 129, 132  
 Sebrell, W. H., 134, 144  
 Shaffner, C. S., 59  
 Shafrir, E., 120, 124  
 Shapiro, R., 156, 162  
 Shepherd, D. B., 119, 123  
 Shepherd, D. M., 283-5  
 Sherwood, S. L., 279, 285  
 Siefert, E., 303-10  
 Siegel, H. S., 104-12  
 Silva, R. B., 140, 144  
 Sinha, M. P., 302-10  
 Siva Sanker, D. V., 141-4  
 Sizer, I. W., 162  
 Slack, E., 201, 202  
 Slover, J., 104, 112  
 Smetana, K., 131, 132  
 Smith, A. H., 73, 74, 134, 141-5  
 Smith, A. J., 238, 241  
 Smith, E. L., 120, 123  
 Smith, G. C., 200, 202  
 Smith, G. H., 156-62  
 Snapir, N., 55-9
- Snook, J. T., 148, 153  
 Sobel, H., 53, 59  
 Sokal, J. E., 119, 123  
 Soliman, H. A., 201, 202  
 Solomon, J. B., 128, 132  
 Somer, P. De, *cf.* De Somer, P.  
 Somogyi, M., 105, 111  
 Soskin, S., 88, 101  
 Spanner, R., 287, 289, 293  
 Sperber, I., 286-93  
 Squance, E., 146-53  
 Stabrowski, E. M., 283-5  
 Stafford, A., 272-3  
 Stahl, H., 212, 226  
 Steele, R., 120, 122  
 Steinberg, D., 119-24  
 Stephenson, M. L., 129, 132  
 Sterlini, J. M., 135, 144  
 Stillman, N., 88, 101  
 St. John, J. L., 147, 154  
 Strookman, T. A., 105, 111  
 Struck, J., 162  
 Stübel, H., 303-10  
 Sturkie, P. D., 113, 124, 193-8, 302-10  
 Sugihara, T. F., 140, 145  
 Sunde, M. L., 164, 170  
 Svatek, E., 105, 112  
 Svendsen, R., 24, 29  
 Sykes, A. H., 11-22, 197-8, 286-93
- Tanaka, K., 12, 13  
 Tarrant, M. E., 115, 124  
 Taylor, F. B., 35, 38  
 Taylor, B. R., 163-70  
 Taylor, M. W., 84, 86  
 Taylor, N. B., 309, 310  
 Taylor, T. G., 182, 199-202  
 Theysen, J. H., 64, 74  
 Theis, H. W., 141-4  
 Thommen, H., 77, 86  
 Thompson, C. R., 76, 86  
 Thompson, R. H. S., 115, 124  
 Thorn, N. A., 64, 74  
 Thornton, P. A., 57, 60  
 Thumin, A., 80, 83, 86  
 Tienhoven, Van A., *cf.* Van Tienhoven, A.  
 Tiews, J., 77, 86  
 Tindall, V. R., 296, 301  
 Timonen, S., 35, 38  
 Titus, H. W., 57, 60, 212, 226  
 Tsuji, F. I., 90, 102  
 Turner, C. W., 53, 59, 60  
 Twombly, J., 148, 154  
 Tyler, C., 203-8
- Urist, M. R., 104, 112, 200, 202  
 Using, H. H., 64, 72, 74  
 Uwaegebute, H. O., 171-80

- van Albada, M., *cf.* Albada, M. van  
 Vande Wiele, R. L., 36, 37  
 Van Drimmelen, G. C., 40, 43  
 Vane, J. R., 268, 273  
 Van Krey, H. P., 39-43  
 Van Tienhoven, A., 5, 7, 10  
 Varley, H., 105, 112  
 Vaughan, M., 120, 124  
 Verdy, M., 120, 123  
 Verzar, F., 275, 278  
 Vogt, M., 281, 285  
 von Saalfeld, E., *cf.* Saalfeld, E. von  
 von Euler, U. S., *cf.* Euler, U. S. von  
 Vuylsteke, C. A., 122, 124  
  
 Waelsch, H., 279, 285  
 Wagner, M., 182, 188  
 Walker, H. A., 84, 86  
 Walpole, A. L., 14, 22  
 Ward, R. T., 131, 132  
 Waring, J. J., 150, 153  
 Warren, D. C., 138, 145  
 Watson, A., 303-10  
 Wattenberg, L. W., 31, 38  
 Watts, A. B., 147, 153  
 Wawrzyniak, M., 57, 60  
 Weiner, N., 119, 123  
 Weliky, I., 35, 38  
 Werbin, H., 35, 37  
 Werner, C., 212, 226  
 West, G. B., 119, 123, 283-5  
 White, A., 104, 112  
 Wiele, R. L. Vande, *cf.* Vande Wiele, R. L.  
 Wilkinson, W. S., 147, 153  
 Williams, A. P., 184, 187  
 Williams, D. J. Jayne-, *cf.* Jayne-Williams,  
 D. J.  
  
 Williams, H. H., 90, 100, 102  
 Williams, J., 135, 145  
 Willis, J. B., 65, 74  
 Willstätter, R., 75, 86  
 Wilson, W., 242, 245  
 Wilson, W. O., 73, 74  
 Windhager, E. E., 72-4  
 Winget, C. M., 134, 141, 145  
 Winter, A. R., 103, 111, 140, 144  
 Wintrobe, W. M., 114, 124  
 Winterstein, A., 75, 86  
 Wirsen, C., 120, 123  
 Wiss, O., 77, 86  
 Wolfe, H. R., 103, 112  
 Wolford, J. H., 104, 109-12  
 Wood-Gush, D. G. M., 11-13, 53, 60  
 Woods, W. D., 147, 153  
 Wostmann, B. S., 182, 188  
 Wright, P. H., 115, 124  
 Wyburn, G. M., 30-8  
  
 Young, A., 296, 301  
  
 Zak, B., 105, 112  
 Zamecnik, P. C., 129, 132  
 Zarrow, I. G., 104, 110, 111  
 Zarrow, M. X., 104, 110, 111  
 Zetterquist, H., 31, 38  
 Zileli, M., 119, 123  
 Zlatis, A., 105, 112  
 Zubay, G., 126, 132  
 Zaimis, E., 249, 260, 279, 285  
 Zander, R., 303-10  
 Zeuthen, E., 302-10  
 Zucker, H., 230, 234



# INDEX OF SUBJECTS

- Acetone drying, of anterior pituitaries, 15-16, 20
- Acetylcholine, 249-53, 263-71, 274-8, 279
- Action potentials, 249-50, 275
- Active transport, 64, 72, 182
- Adenohypophysis (anterior pituitary), (*cf.* Hypophysis, Pituitary), 3-15, 53, 179, 201
- Adenosine tri-phosphate (ATP), 125, 30
- Adipose tissue, release of FFA from, 113, 119-22
- Adrenal, 36, 46, 50, 103-10, 110, 279-83
- Adrenergic stimulation, 116
- Adrenaline (*cf.* Catecholamines)
- Adrenocorticotrophic Hormone (ACTH), 103-11
- Albumin, 137-43
- Amibenonium, methoxyamibenonium, 257-258, 264
- Amino-acids (*cf.* proteins)  
in oviduct, 131, 125-31, 134, 141  
in diet, 155-80
- Aminopeptidase, 13
- Amnion, 274-8, plate 17
- Ammonia  
faecal, 147  
gaseous, 237-8
- Anastomoses, 193-5
- Androgens (*cf.* Testosterone), 199-200
- Androstenediol (*cf.* Hydroxysteroids)
- Antibiotics, 185-7
- Anticholinesterases, 249-59, 263-6, 274-8
- Apocrine, 45
- Arachidonic acid, 87-99
- Arginine, 156-62
- Artificial insemination (A.I.) (*cf.* Insemination), 39-42, 52-9, 88
- Ascorbic acid (AA)  
effect on semen quality, 52-8  
in adrenals, 103-11
- Atomic absorption spectroscopy (AAS), 65
- Atresia (*cf.* Follicle), 20, 36, 68
- Atropine, 263-71, 277, 290
- Availability, of amino acids (*cf.* Digestibility), 171-80
- Avidin, 134-43
- Batteries, 53-5, 245
- Benzoquinonium, 251-5
- Blastodisc, 42
- Blood  
sugar, 103-11, 228  
supply to kidney, 286-92  
supply to liver, 286, 294-300  
supply to shellgland, 191-8
- Blubber-adrenal barrier, 281
- Blubber-adrenal barrier, 277-84
- Bradykinin, 271
- Brain, catecholamine content of, 277-83
- Breathing (*cf.* respiration)
- Diethylmercuric acid diethylamide, 268
- Diethylstilbestrol, 274-6
- Notes of Palaeoecia, 103-11, 182
- Calomycin, 201
- Calcium, 11-13, 48, 63-73, 197, 199-200, 273
- Capillary, 207
- Cannulation (*cf.* Catheterisation)  
shellgland wall, 197  
nerves, 307
- Carbachol, 263-4, 270
- Carbohydrate metabolism, effect of ACTH, 111
- Carbolonium (imibertil), 249
- Carbon 14 ( $C^{14}$ ), 23-8, 114, 129
- Carbon dioxide  
plasma, 47-8, 197-8  
atmospheric, 211, 218  
respiratory, 212-23
- Carotenoids, 75-85, 183, plate 12
- Catabolism, of amino acids, 160
- Catecholamines, 119-22, 263-71, 279-85, 290
- Catheterisation (*cf.* cannulation),  
of ureter, 286  
of jugular vein, 296
- Central nervous system, 258-9, 279
- Chalazae, 140
- Chloride  
in seminal plasma, 48  
uptake into brain, 279
- Cholesterol (DHA sulphate) (*cf.* Hydroxysteroids), 87-91, 97-100, 103-10, 181-3
- Choline synthesis, 88
- Cholinesterases, 249-53, 255-8, 264, 274-8  
active sites, 257-8  
distribution in amnion, 275-6  
inhibition of, 253-5
- Chromatography, 135  
alumina, 24-6, 78-9, 105  
column, 78, 97, 131, 138-40  
gas, 47-8  
thin layer (TLC), 24-6  
paper, 23
- Clearance,  
BSP, 294-300  
 $C^{14}$  palmitate, 114  
renal, 287-90
- Cloaca, 44-6, 146

- Clostridium welchii*, 187
- Clutch, 4, 8-9, 12
- Cocaine, 12
- Coccygeo-mesenteric vein, 286-98
  - ligation of, 297-300
- Colostomy, 146-52
- Comb size, 14, 18
- Conalbumin, 134-43
- Consumer acceptability (yolk colour), 77
- Contraction, spontaneous, of amnion, 274-5
- Cortisol, Cortisone, 37
- Creatinine, 147, 289
- Crista, 35
- Crop, 261-72
  - response to stimulation, 270
  - sensitivity to 5-HT, 271-2
- Crop tube (force feeding), 81
- Cross-figures, 274-6, plate 17
- Cryptoxanthin, 75-6
- Cytoplasm, of ovum, 30-2
  
- Decamethonium, 250, 255
- Depolarising drugs, 249-59
- Dibenamine, 6, 7
- Diets, 150, 157, 165, 167, 173, 238, 240
  - all-vegetable protein, 88
  - basal, 157, 159, 165, 173, 174, 176
  - Ca-deficient, 201
  - low-pigment, 79-80
  - nitrogen free, 146, 148
- Digestibility, true and apparent, 146-50
- Digtonin precipitation of carotene contaminant, 78
- DMPP, 264
  
- Electron microscope, 30-4, 131, plates 2-5, 13-15
- Electrophoresis, 133-42
- Embryo
  - cholinesterase in, 276
  - lipid metabolism of, 87-101
  - potassium needs of, 70
  - protein synthesis in, 126
  - vitamin B<sub>12</sub> deficient, 88
- Embryonic mortality, 39-43
- Endocrine control systems, 3, 199-202
- Endoplasmic reticulum, 33-5, 125-6, 131
- Environment, 211-45
  - daylight hours, 54-5, 240
  - humidity, 219-26
  - temperature, 54-6, 211-26, 228-33
- Enzymes
  - in protein synthesis, 125, 128
  - in steroid metabolism, 30-1
  - in yolk lipid degradation, 99
  - digestive, 148, 151
  - pancreatic, 181-3
  - acid phosphatase, 50
  - cholinesterases, 249-59, 274-6
  - lecithinase C, 130
  - ribonuclease, 130
- Epididymis, 44-9
- Eserine, 274, 277
- Erectile structures, 44-7
- Excreta (*cf.* Urine, Faeces)
- Excretion
  - differential, 287-9
  - kidney, 286-90
  - vas deferens, 45, 50

# INDEX OF SUBJECTS

- Acetone drying, of anterior pituitaries,  
15-16, 20
- Acetylcholine, 249-58, 263-71, 274-8, 289
- Action potentials, 249-50, 275
- Active transport, 64, 72, 182
- Adenohypophysis (anterior pituitary), (*cf.*  
Hypophysis, Pituitary), 3-15, 53, 199,  
201
- Adenosine tri-phosphate (ATP), 125-30
- Adipose tissue, release of FFA from, 113,  
119-22
- Adrenal, 36, 46, 59, 103-10, 119, 279-83
- Adrenergic stimulation, 116
- Adrenaline (*cf.* Catecholamines)
- Adrenocorticotrophic Hormone (ACTH),  
103-11
- Albumin, 137-43
- Ambenonium, methoxyambenonium, 252-  
255, 264
- Amino-acids (*cf.* proteins)  
in oviduct, 88, 125-31, 134, 141  
in diet, 155-80
- Aminopeptidase, 13
- Amnion, 274-8, plate 17
- Ammonia  
faecal, 147  
gaseous, 237-8
- Anastomoses, 193-5
- Androgens (*cf.* Testosterone), 199-200
- Androstenediol (*cf.* Hydroxysteroids)
- Antibiotics, 185-7
- Anticholinesterases, 249-59, 263-6, 274-8
- Apocrine, 45
- Arachidonic acid, 87-99
- Arginine, 156-62
- Artificial insemination (A.I.) (*cf.* Insemina-  
tion), 39-42, 52-9, 88
- Ascorbic acid (AA)  
effect on semen quality, 52-8  
in adrenals, 103-11
- Atomic absorption spectroscopy (AAS), 65
- Atresia (*cf.* Follicle), 20, 36, 68
- Atropine, 263-71, 277, 290
- Availability, of amino acids (*cf.* Digestibility),  
171-80
- Avidin, 134-45
- Batteries, 53-5, 245
- Benzoquinonium, 251-3
- Blastodisc, 42
- Blood  
sugar, 103-11, 228  
supply to kidney, 286-92  
supply to liver, 286, 294-300  
supply to shellgland, 191-2
- Blood-adrenal barrier, 281
- Blood-brain barrier, 279-84
- Bradykinin, 271
- Brain, catecholamine content of, 279-83
- Breathing (*cf.* respiration)
- Bromolysergic acid diethylamide, 268
- Bromsulphthalein, 294-6
- Bursa of Fabricius, 103-11, 182
- Calcitonin, 201
- Calcium, 11-13, 48, 63-73, 197, 199-202,  
278
- Candling, 207
- Cannulation (*cf.* Catheterisation)  
shellgland wall, 197  
trachea, 307
- Carbachol, 263-4, 290
- Carbohydrate metabolism, effect of ACTH,  
111
- Carbolonium (imbretil), 249
- Carbon 14 ( $C^{14}$ ), 23-8, 114, 129
- Carbon dioxide  
plasma, 47-8, 197-8  
atmospheric, 211, 218  
respiratory, 212-23
- Carotenoids, 75-85, 183, plate 12
- Catabolism, of amino acids, 160
- Catecholamines, 119-22, 263-71, 279-85,  
290
- Catheterisation (*cf.* cannulation),  
of ureter, 286  
of jugular vein, 296
- Central nervous system, 258-9, 279
- Chalazae, 140
- Chloride  
in seminal plasma, 48  
uptake into brain, 279
- Cholesterol (DHA sulphate) (*cf.* Hydroxy-  
steroids), 87-91, 97-100, 103-10, 181-5
- Choline synthesis, 89
- Cholinesterases, 249-53, 255-8, 264, 274-8  
active sites, 257-8  
distribution in amnion, 275-6  
inhibition of, 253-5
- Chromatography, 135  
alumina, 24-6, 78-9, 105  
column, 78, 97, 131, 133-40  
gas, 47-8  
thin layer (TLC), 24-6  
paper, 23
- Clearance,  
HSP, 294-300  
 $C^{14}$  palmitate, 114  
renal, 287-90
- Clouza, 44-6, 146

# INDEX OF SUBJECTS

- Acetone drying, of anterior pituitaries, 15-16, 20
- Acetylcholine, 249-58, 263-71, 274-8, 289
- Action potentials, 249-50, 275
- Active transport, 64, 72, 182
- Adenohypophysis (anterior pituitary), (*cf.* Hypophysis, Pituitary), 3-15, 53, 199, 201
- Adenosine tri-phosphate (ATP), 125-30
- Adipose tissue, release of FFA from, 113, 119-22
- Adrenal, 36, 46, 59, 103-10, 119, 279-83
- Adrenergic stimulation, 116
- Adrenaline (*cf.* Catecholamines)
- Adrenocorticotrophic Hormone (ACTH), 103-11
- Albumin, 137-43
- Ambenonium, methoxyambenonium, 252-255, 264
- Amino-acids (*cf.* proteins)  
in oviduct, 88, 125-31, 134, 141  
in diet, 155-80
- Aminopeptidase, 13
- Amnion, 274-8, plate 17
- Ammonia  
faecal, 147  
gaseous, 237-8
- Anastomoses, 193-5
- Androgens (*cf.* Testosterone), 199-200
- Androstenediol (*cf.* Hydroxysteroids)
- Antibiotics, 185-7
- Anticholinesterases, 249-59, 263-6, 274-8
- Apocrine, 45
- Arachidonic acid, 87-99
- Arginine, 156-62
- Artificial insemination (A.I.) (*cf.* Insemination), 39-42, 52-9, 88
- Ascorbic acid (AA)  
effect on semen quality, 52-8  
in adrenals, 103-11
- Atomic absorption spectroscopy (AAS), 65
- Atresia (*cf.* Follicle), 20, 36, 68
- Atropine, 263-71, 277, 290
- Availability, of amino acids (*cf.* Digestibility), 171-80
- Avidin, 134-43
- Batteries, 53-5, 245
- Benzoquinonium, 251-5
- Blastodisc, 42
- Blood  
sugar, 103-11, 228  
supply to kidney, 286-92  
supply to liver, 286, 294-300  
supply to shellgland, 191-8
- Blood-adrenal barrier, 281
- Blood-brain barrier, 279-84
- Bradykinin, 271
- Brain, catecholamine content of, 279-83
- Breathing (*cf.* respiration)
- Bromolysergic acid diethylamide, 268
- Bromsulphthalein, 294-6
- Bursa of Fabricius, 103-11, 182
- Calcitonin, 201
- Calcium, 11-13, 48, 63-73, 197, 199-202, 278
- Candling, 207
- Cannulation (*cf.* Catheterisation)  
shellgland wall, 197  
trachea, 307
- Carbachol, 263-4, 290
- Carbohydrate metabolism, effect of ACTH, 111
- Carbolonium (imbretil), 249
- Carbon 14 ( $C^{14}$ ), 23-8, 114, 129
- Carbon dioxide  
plasma, 47-8, 197-8  
atmospheric, 211, 218  
respiratory, 212-23
- Carotenoids, 75-85, 183, plate 12
- Catabolism, of amino acids, 160
- Catecholamines, 119-22, 263-71, 279-85, 290
- Catheterisation (*cf.* cannulation),  
of ureter, 286  
of jugular vein, 296
- Central nervous system, 258-9, 279
- Chalazae, 140
- Chloride  
in seminal plasma, 48  
uptake into brain, 279
- Cholesterol (DHA sulphate) (*cf.* Hydroxysteroids), 87-91, 97-100, 103-10, 181-5
- Choline synthesis, 88
- Cholinesterases, 249-53, 255-8, 264, 274-8  
active sites, 257-8  
distribution in amnion, 275-6  
inhibition of, 253-5
- Chromatography, 135  
alumina, 24-6, 78-9, 105  
column, 78, 97, 131, 138-40  
gas, 47-8  
thin layer (TLC), 24-6  
paper, 23
- Clearance,  
BSP, 294-300  
 $C^{14}$  palmitate, 114  
renal, 287-90
- Cloaca, 44-6, 146

- Formazan (mono-, di-), 31-6  
 Free (unesterified) fatty acids (FFA), 99, 113-22  
 Freeze-dried ACTH, 104  
   pituitary, 20  
 Freezing-point depression  
   cock plasma, 70, 73  
   egg white, 70-73  
   seminal fluid, 48
- Gaseous exchange (*cf.* oxygen, carbon dioxide), 211, 214, 219  
 Gasping response, 119  
 Gastrocnemius muscle preparation, 249-50  
 Germ-free chickens, 181-7  
   characteristics, 182-3  
 Glomerular filtration, 287-9  
 Glucagon, 113-22  
 Glucocorticoid synthesis, 111  
 Glucose, 113-5  
 Glutamate, glutamic acid, 48, 174-5  
 Glutaraldehyde, and osmic acid fixation, 31  
 Glycogenesis, 103-110, 115  
 Golgi substance, 32, 35  
 Gonads  
   Dependence on hypophysial secretion, 3  
   Possible effect of ascorbic acid on, 58  
 $\beta$  Gonadotrophic cells (anterior hypophysis), 53  
 Gonadotrophin, 14-22, 199-202  
 Granulosa (follicle), 30-7  
 Growth rate, 156, 161, 171-3, 186-7, 171-3, 229-33, 240  
 Guanethidine, 279-84  
 Guanosine triphosphate (GTP), 126-30
- Hatchability (*cf.* Fertility), 63, 73, 88  
 Heart, catecholamine content of, 279-83  
 Heart puncture, 4-5, 105  
 Heat conservation, 229  
 Heat increment of food, 217, 226  
 Heat production, 211-4, 217-26, 233  
 Heat tolerance, 225-6  
 Hemicholinium, 266  
 Hering-Breuer reflexes, 309  
 Hexamethonium, 113-6, 120, 262-6, 271  
 Histamine, 263-71, 287-9  
 Histidine, 157  
 Holocrine, 45  
 Homeostasis, 170, 231  
 Hormones (*cf.* specific names), 12, 14-22, 30, 59, 113-22, 133-43, 199-202  
 Housing, 212-3, 219-21, 235-45  
   heating, 235, 241-5  
   insulation, 212-3, 235, 241-3  
   ventilation, 211-3, 218, 222-6, 235, 241  
 Huddling, 228-9, 233  
 Humidity, 211-2, 219, 221-6, 242  
 Hydrogen ion concentration (*cf.* pH)
- Hydroxysteroids, 31-6  
 Hydroxyllysine, 179  
 Hydroxysteroid dehydrogenases, 30-1  
 5-Hydroxytryptamine (5HT), 261-72  
 Hypercalcaemia, 199-201  
 Hyperglycaemia, 103-11, 122  
 Hyperplastic adrenal homogenates, 36  
 Hypophysectomy, 3, 5, 14-22  
 Hypophysis (*cf.* Adenohypophysis, Pituitary) 3-9, 53  
 Hypothalamus, 3, 156, 199, 201, 281
- I.C.I. Compound 33828, 14-22  
 Indian ink, injection of shell gland vascular system, 191-5  
 Indocyanine green (ICG), 297  
 Induction, of enzyme formation, 276  
 Infundibulum (funnel), 39-42  
   water-soluble protein content of, 139  
 Insecticides, 249, 258-9  
 Insemination, 39-42, 52-9  
 Insulin, 113-22  
 Inulin (clearance), 287  
 Ionic exchange (Na-K), 69  
 "I" particles, 125-31, plates 13-15  
 Isoleucine, 156, 166-8  
 Isotopes, radioactive, 125-30  
   Cl, 279  
   C<sup>14</sup>, 23-9  
   <sup>35</sup>S, 149, 164  
   T, 23-9  
 Isthmus, 7, 65, 69-71  
   water soluble proteins in, 139
- Kidney, venous system and excretion of, (*cf.* Nephron) 286-92  
 Kinoplasmic droplet, 44, 49  
 Kossin test, 42  
 Krebs's solution, 261-3, 271
- Laparotomy, 17, 21, 41  
 "Layers' cramp," 199, 202  
 Laying Cycle (*cf.* Ovulatory Cycle)  
 Lecithase, 99, 130  
 Lecithin, 88  
 Leucine, 156, 166-8  
 Leydig cells, 36  
 Libido, 53, 57  
 Linoleic acid, 93, 97  
 Lipase, 99  
 Lipids, 87-101, 113-4  
   counting, 26-7

- Formazan (mono-, di-), 31-6  
 Free (unesterified) fatty acids (FFA), 99, 113-22  
 Freeze-dried ACTH, 104  
   pituitary, 20  
 Freezing-point depression  
   cock plasma, 70, 73  
   egg white, 70-73  
   seminal fluid, 48
- Gaseous exchange (*cf.* oxygen, carbon dioxide), 211, 214, 219  
 Gasping response, 119  
 Gastrocnemius muscle preparation, 249-50  
 Germ-free chickens, 181-7  
   characteristics, 182-3  
 Glomerular filtration, 287-9  
 Glucagon, 113-22  
 Glucocorticoid synthesis, 111  
 Glucose, 113-5  
 Glutamate, glutamic acid, 48, 174-5  
 Glutaraldehyde, and osmic acid fixation, 31  
 Glycogenesis, 103-110, 115  
 Golgi substance, 32, 35  
 Gonads  
   Dependence on hypophyseal secretion, 3  
   Possible effect of ascorbic acid on, 58  
 $\beta$  Gonadotrophic cells (anterior hypophysis), 53  
 Gonadotrophin, 14-22, 199-202  
 Granulosa (follicle), 30-7  
 Growth rate, 156, 161, 171-3, 186-7, 171-3, 229-33, 240  
 Guanethidine, 279-84  
 Guanosine triphosphate (GTP), 126-30
- Hatchability (*cf.* Fertility), 63, 73, 88  
 Heart, catecholamine content of, 279-83  
 Heart puncture, 4-5, 105  
 Heat conservation, 229  
 Heat increment of food, 217, 226  
 Heat production, 211-4, 217-26, 233  
 Heat tolerance, 225-6  
 Hemicholinium, 266  
 Hering-Breuer reflexes, 309  
 Hexamethonium, 113-6, 120, 262-6, 271  
 Histamine, 263-71, 287-9  
 Histidine, 157  
 Holocrine, 45  
 Homeostasis, 170, 231  
 Hormones (*cf.* specific names), 12, 14-22, 30, 59, 113-22, 133-43, 199-202  
 Housing, 212-3, 219-21, 235-45  
   heating, 235, 241-5  
   insulation, 212-3, 235, 241-3  
   ventilation, 211-3, 218, 222-6, 235, 241  
 Huddling, 228-9, 233  
 Humidity, 211-2, 219, 221-6, 242  
 Hydrogen ion concentration (*cf.* pH)
- Hydroxysteroids, 31-6  
 Hydroxylysine, 179  
 Hydroxysteroid dehydrogenases, 30-1  
 5-Hydroxytryptamine (5HT), 261-72  
 Hypercalcaemia, 199-201  
 Hyperglycaemia, 103-11, 122  
 Hyperplastic adrenal homogenates, 36  
 Hypophysectomy, 3, 5, 14-22  
 Hypophysis (*cf.* Adenohypophysis, Pituitary) 3-9, 53  
 Hypothalamus, 3, 156, 199, 201, 281
- I.C.I. Compound 33828, 14-22  
 Indian ink, injection of shell gland vascular system, 191-5  
 Indocyanine green (ICG), 297  
 Induction, of enzyme formation, 276  
 Infundibulum (funnel), 39-42  
   water-soluble protein content of, 139  
 Insecticides, 249, 258-9  
 Insemination, 39-42, 52-9  
 Insulin, 113-22  
 Inulin (clearance), 287  
 Ionic exchange (Na-K), 69  
 "I" particles, 125-31, plates 13-15  
 Isoleucine, 156, 166-8  
 Isotopes, radioactive, 125-30  
   Cl, 279  
   C<sup>14</sup>, 23-9  
   <sup>35</sup>S, 149, 164  
   T, 23-9  
 Isthmus, 7, 65, 69-71  
   water soluble proteins in, 139
- Kidney, venous system and excretion of, (*cf.* Nephron) 286-92  
 Kinoplasmic droplet, 44, 49  
 Kossin test, 42  
 Krebs's solution, 261-3, 271
- Laparotomy, 17, 21, 41  
 "Layers' cramp," 199, 202  
 Laying Cycle (*cf.* Ovarulatory Cycle)  
 Lecithase, 99, 130  
 Lecithin, 88  
 Leucine, 156, 166-8  
 Leydig cells, 36  
 Libido, 53, 57  
 Linoleic acid, 93, 97  
 Lipase, 99  
 Lipids, 87-101, 113-4  
 Liquid scintillation counting, 26-7  
 Liver  
   lipids in, 87-101, 113  
   glycogenesis, 105, 108  
   oxygen uptake, 160  
 Luteal cells, 32, 37

- Formazan (mono-, di-), 31-6  
 Free (unesterified) fatty acids (FFA), 99, 113-22  
 Freeze-dried ACTH, 104  
   pituitary, 20  
 Freezing-point depression  
   cock plasma, 70, 73  
   egg white, 70-73  
   seminal fluid, 48
- Gaseous exchange (*cf.* oxygen, carbon dioxide), 211, 214, 219  
 Gasping response, 119  
 Gastrocnemius muscle preparation, 249-50  
 Germ-free chickens, 181-7  
   characteristics, 182-3  
 Glomerular filtration, 287-9  
 Glucagon, 113-22  
 Glucocorticoid synthesis, 111  
 Glucose, 113-5  
 Glutamate, glutamic acid, 48, 174-5  
 Glutaraldehyde, and osmic acid fixation, 31  
 Glycogenesis, 103-110, 115  
 Golgi substance, 32, 35  
 Gonads  
   Dependence on hypophysial secretion, 3  
   Possible effect of ascorbic acid on, 58  
 $\beta$  Gonadotrophic cells (anterior hypophysis), 53  
 Gonadotrophin, 14-22, 199-202  
 Granulosa (follicle), 30-7  
 Growth rate, 156, 161, 171-3, 186-7, 171-3, 229-33, 240  
 Guanethidine, 279-84  
 Guanosine triphosphate (GTP), 126-30
- Hatchability (*cf.* Fertility), 63, 73, 88  
 Heart, catecholamine content of, 279-83  
 Heart puncture, 4-5, 105  
 Heat conservation, 229  
 Heat increment of food, 217, 226  
 Heat production, 211-4, 217-26, 233  
 Heat tolerance, 225-6  
 Hemicholinium, 266  
 Hering-Breuer reflexes, 309  
 Hexamethonium, 113-6, 120, 262-6, 271  
 Histamine, 263-71, 287-9  
 Histidine, 157  
 Holocrine, 45  
 Homeostasis, 170, 231  
 Hormones (*cf.* specific names), 12, 14-22, 30, 59, 113-22, 133-43, 199-202  
 Housing, 212-3, 219-21, 235-45  
   heating, 235, 241-5  
   insulation, 212-3, 235, 241-3  
   ventilation, 211-3, 218, 222-6, 235, 241  
 Huddling, 228-9, 233  
 Humidity, 211-2, 219, 221-6, 242  
 Hydrogen ion concentration (*cf.* pH)
- Hydroxysteroids, 31-6  
 Hydroxylysine, 179  
 Hydroxysteroid dehydrogenases, 30-1  
 5-Hydroxytryptamine (5HT), 261-72  
 Hypercalcaemia, 199-201  
 Hyperglycaemia, 103-11, 122  
 Hyperplastic adrenal homogenates, 36  
 Hypophysectomy, 3, 5, 14-22  
 Hypophysis (*cf.* Adenohypophysis, Pituitary) 3-9, 53  
 Hypothalamus, 3, 156, 199, 201, 281
- I.C.I. Compound 33828, 14-22  
 Indian ink, injection of shell gland vascular system, 191-5  
 Indocyanine green (ICG), 297  
 Induction, of enzyme formation, 276  
 Infundibulum (funnel), 39-42  
   water-soluble protein content of, 139  
 Insecticides, 249, 258-9  
 Insemination, 39-42, 52-9  
 Insulin, 113-22  
 Inulin (clearance), 287  
 Ionic exchange (Na-K), 69  
 "I" particles, 125-31, plates 13-15  
 Isoleucine, 156, 166-8  
 Isotopes, radioactive, 125-30  
   Cl, 279  
   C<sup>14</sup>, 23-9  
   <sup>35</sup>S, 149, 164  
   T, 23-9  
 Isthmus, 7, 65, 69-71  
   water soluble proteins in, 139
- Kidney, venous system and excretion of, (*cf.* Nephron) 286-92  
 Kinoplasmic droplet, 44, 49  
 Kossin test, 42  
 Krebs's solution, 261-3, 271
- Laparotomy, 17, 21, 41  
 "Layers' cramp," 199, 202  
 Laying Cycle (*cf.* Ovulatory Cycle)  
 Lecithase, 99, 130  
 Lecithin, 88  
 Leucine, 156, 166-8  
 Leydig cells, 36  
 Libido, 53, 57  
 Linoleic acid, 93, 97  
 Lipase, 99  
 Lipids, 87-101, 113-4  
 Liquid scintillation counting, 26-7  
 Liver  
   lipids in, 87-101, 113  
   glycogenesis, 105, 108  
   oxygen uptake, 160  
 Luteal cells, 32, 37

- Formazan (mono-, di-), 31-6  
 Free (unesterified) fatty acids (FFA), 99, 113-22  
 Freeze-dried ACTH, 104  
   pituitary, 20  
 Freezing-point depression  
   cock plasma, 70, 73  
   egg white, 70-73  
   seminal fluid, 48
- Gaseous exchange (*cf.* oxygen, carbon dioxide), 211, 214, 219  
 Gasping response, 119  
 Gastrocnemius muscle preparation, 249-50  
 Germ-free chickens, 181-7  
   characteristics, 182-3  
 Glomerular filtration, 287-9  
 Glucagon, 113-22  
 Glucocorticoid synthesis, 111  
 Glucose, 113-5  
 Glutamate, glutamic acid, 48, 174-5  
 Glutaraldehyde, and osmic acid fixation, 31  
 Glycogenesis, 103-110, 115  
 Golgi substance, 32, 35  
 Gonads  
   Dependence on hypophyseal secretion, 3  
   Possible effect of ascorbic acid on, 58  
 $\beta$  Gonadotrophic cells (anterior hypophysis), 53  
 Gonadotrophin, 14-22, 199-202  
 Granulosa (follicle), 30-7  
 Growth rate, 156, 161, 171-3, 186-7, 171-3, 229-33, 240  
 Guanethidine, 279-84  
 Guanosine triphosphate (GTP), 126-30
- Hatchability (*cf.* Fertility), 63, 73, 88  
 Heart, catecholamine content of, 279-83  
 Heart puncture, 4-5, 105  
 Heat conservation, 229  
 Heat increment of food, 217, 226  
 Heat production, 211-4, 217-26, 233  
 Heat tolerance, 225-6  
 Hemicholinium, 266  
 Hering-Breuer reflexes, 309  
 Hexamethonium, 113-6, 120, 262-6, 271  
 Histamine, 263-71, 287-9  
 Histidine, 157  
 Holoerine, 45  
 Homeostasis, 170, 231  
 Hormones (*cf.* specific names), 12, 14-22, 30, 59, 113-22, 133-43, 199-202  
 Housing, 212-3, 219-21, 235-45  
   heating, 235, 241-5  
   insulation, 212-3, 235, 241-3  
   ventilation, 211-3, 218, 222-6, 235, 241  
 Huddling, 228-9, 233  
 Humidity, 211-2, 219, 221-6, 242  
 Hydrogen ion concentration (*cf.* pH)
- Hydroxysteroids, 31-6  
 Hydroxylysine, 179  
 Hydroxysteroid dehydrogenases, 30-1  
 5-Hydroxytryptamine (5HT), 261-72  
 Hyperealaemia, 199-201  
 Hyperglycaemia, 103-11, 122  
 Hyperplastic adrenal homogenates, 36  
 Hypophysectomy, 3, 5, 14-22  
 Hypophysis (*cf.* Adenohypophysis, Pituitary) 3-9, 53  
 Hypothalamus, 3, 156, 199, 201, 281
- I.C.I. Compound 33828, 14-22  
 Indian ink, injection of shell gland vascular system, 191-5  
 Indocyanine green (ICG), 297  
 Induction, of enzyme formation, 276  
 Infundibulum (funnel), 39-42  
   water-soluble protein content of, 139  
 Insecticides, 249, 258-9  
 Insemination, 39-42, 52-9  
 Insulin, 113-22  
 Inulin (clearance), 287  
 Ionic exchange (Na-K), 69  
 "I" particles, 125-31, plates 13-15  
 Isoleucine, 156, 166-8  
 Isotopes, radioactive, 125-30  
   Cl, 279  
   C<sup>14</sup>, 23-9  
   <sup>35</sup>S, 149, 164  
   T, 23-9  
 Isthmus, 7, 65, 69-71  
   water soluble proteins in, 139
- Kidney, venous system and excretion of, (*cf.* Nephron) 286-92  
 Kinoplasmic droplet, 44, 49  
 Kossin test, 42  
 Krebs's solution, 261-3, 271
- Laparotomy, 17, 21, 41  
 "Layers' cramp," 199, 202  
 Laying Cycle (*cf.* Ovarulatory Cycle)  
 Lecithase, 99, 130  
 Lecithin, 88  
 Leucine, 156, 166-8  
 Leydig cells, 36  
 Libido, 53, 57  
 Linoleic acid, 93, 97  
 Lipase, 99  
 Lipids, 87-101, 113-4  
 Liquid scintillation counting, 26-7  
 Liver  
   lipids in, 87-101, 113  
   glycogenesis, 105, 108  
   oxygen uptake, 160  
 Luteal cells, 32, 37



- 'ormazan (mono-, di-), 31-6  
 free (unesterified) fatty acids (FFA), 99,  
     113-22  
 Freeze-dried ACTH, 104  
     pituitary, 20  
 Freezing-point depression  
     cock plasma, 70, 73  
     egg white, 70-73  
     seminal fluid, 48
- Gaseous exchange (*cf.* oxygen, carbon  
     dioxide), 211, 214, 219
- Gasping response, 119  
 Gastrocnemius muscle preparation, 249-50  
 Germ-free chickens, 181-7  
     characteristics, 182-3  
 Glomerular filtration, 287-9  
 Glucagon, 113-22  
 Glucocorticoid synthesis, 111  
 Glucose, 113-5  
 Glutamate, glutamic acid, 48, 174-5  
 Glutaraldehyde, and osmic acid fixation, 31  
 Glycogenesis, 103-110, 115  
 Golgi substance, 32, 35  
 Gonads  
     Dependence on hypophysial secretion, 3  
     Possible effect of ascorbic acid on, 58  
 $\beta$  Gonadotrophic cells (anterior hypophysis),  
     53  
 Gonadotrophin, 14-22, 199-202  
 Granulosa (follicle), 30-7  
 Growth rate, 156, 161, 171-3, 186-7, 171-3,  
     229-33, 240  
 Guanethidine, 279-84  
 Guanosine triphosphate (GTP), 126-30
- Hatchability (*cf.* Fertility), 63, 73, 88  
 Heart, catecholamine content of, 279-83  
 Heart puncture, 4-5, 105  
 Heat conservation, 229  
 Heat increment of food, 217, 226  
 Heat production, 211-4, 217-26, 233  
 Heat tolerance, 225-6  
 Hemicholinium, 266  
 Hering-Breuer reflexes, 309  
 Hexamethonium, 113-6, 120, 262-6, 271  
 Histamine, 263-71, 287-9  
 Histidine, 157  
 Holocrine, 45  
 Homeostasis, 170, 231  
 Hormones (*cf.* specific names), 12, 14-22,  
     30, 59, 113-22, 133-43, 199-202  
 Housing, 212-3, 219-21, 235-45  
     heating, 235, 241-5  
     insulation, 212-3, 235, 241-3  
     ventilation, 211-3, 218, 222-6, 235, 241  
 Huddling, 228-9, 233  
 Humidity, 211-2, 219, 221-6, 242  
 Hydrogen ion concentration (*cf.* pH)
- Hydroxysteroids, 31-6  
 Hydroxylysine, 179  
 Hydroxysteroid dehydrogenases, 30-1  
 5-Hydroxytryptamine (5HT), 261-72  
 Hypercalcaemia, 199-201  
 Hyperglycaemia, 103-11, 122  
 Hyperplastic adrenal homogenates, 36  
 Hypophysectomy, 3, 5, 14-22  
 Hypophysis (*cf.* Adenohypophysis, Pituitary)  
     3-9, 53  
 Hypothalamus, 3, 156, 199, 201, 281
- I.C.I. Compound 33828, 14-22  
 Indian ink, injection of shell gland vascular  
     system, 191-5  
 Indocyanine green (ICG), 297  
 Induction, of enzyme formation, 276  
 Infundibulum (funnel), 39-42  
     water-soluble protein content of, 139  
 Insecticides, 249, 258-9  
 Insemination, 39-42, 52-9  
 Insulin, 113-22  
 Inulin (clearance), 287  
 Ionic exchange (Na-K), 69  
 "I" particles, 125-31, plates 13-15  
 Isoleucine, 156, 166-8  
 Isotopes, radioactive, 125-30  
     Cl, 279  
     C<sup>14</sup>, 23-9  
     <sup>35</sup>S, 149, 164  
     T, 23-9  
 Isthmus, 7, 65, 69-71  
     water soluble proteins in, 139
- Kidney, venous system and excretion of,  
     (*cf.* Nephron) 286-92  
 Kinoplasmic droplet, 44, 49  
 Kossin test, 42  
 Krebs's solution, 261-3, 271
- Laparotomy, 17, 21, 41  
 "Layers' cramp," 199, 202  
 Laying Cycle (*cf.* Ovulatory Cycle)  
 Lecithase, 99, 130  
 Lecithin, 88  
 Leucine, 156, 166-8  
 Leydig cells, 36  
 Libido, 53, 57  
 Linoleic acid, 93, 97  
 Lipase, 99  
 Lipids, 87-101, 113-4  
 Liquid scintillation counting, 26-7  
 Liver  
     lipids in, 87-101, 113  
     glycogenesis, 105, 108  
     oxygen uptake, 160  
 Luteal cells, 32, 37

- Lutein (*cf.* Xanthophylls)
- Lutein cells, 33
- Luteinising hormone (LH), 3-9
- Lymph folds (*cf.* Erectile structures)
- Lysine, 129, 150, 156-62, 166-8, 173-9
  - available, 175-9
- Lysozyme, 135-43
  
- Magnesium, 48, 63-73
- Magnum, 3, 7, 41, 65-72, 125, 134-43
  - thread in, 7
  - Riboflavin content of, 134
- Mecamylamine, 264-6
- Medullary bone, 199-202
- Membrane, cell (in amnion), 274-8
- Membrane, shell, 69-72, 191
  - effect on shell strength, 203, 207
- Membrane, yolk, 73
- Membrane permeability, 36
- Mepyramine, 269-71
- Metabolism
  - effect of ascorbic acid, on, 57-9
  - energy, 211-26
  - plasma fatty acids, 122
  - respiratory, 215-8, 222-6
- Metabolic Rate, 211-25, 229
  - Basal (BMR), 211, 216-20
- Metabolic faecal nitrogen (MFN), 146-52
- Methionine, 88, 149, 150, 157, 163-4, 237-40
- Michel clips (*cf.* Thread), in shell gland, 12
- Microsomes, comparison of "I" particles
  - with, 125-30
- Minute volume, 304, 309
- Mipafox, 277
- Mitochondria, 33-5, 128, 131, plates 13
  - and 14
- Morphine, 266-8
- Motor end-plates, 249
  
- Oestrogens (oestrone, oestradiol, oestriol),
  - 23-30, 35, 37, 133-43, 199-202
- Oestrogen-androgen synergism, 199
- Oleic acid, 91-2, 98
- Osmic acid fixation, 31
- Osmotic pressure
  - egg white, 63, 70
  - seminal plasma, 44, 50
- Ovalbumin, 131-43
- Ovarian Ascorbic Acid Depletion
  - (O.A.A.D.) method, 3-5, 10
- Ovary (*cf.* Follicle, Ovum), 12, 14-22, 64,
  - 133, 143, 199
- Oviducal eggs, 20, 64-7
- Oviduct, 4-5, 11, 37, 50, 63-5, 71-3
  - effect of hormones on, 14-22, 133-43
  - proteins in, 125-31, 133-43
  - puncture, 41
  - spermatozoa in, 39-43
- Oviposition, 3-5, 11-13, 41, 63, 73
- Ovoglobulin, 136-43
- Ovomucin-lysozyme complex, 72
- Ovomucoid, 136-43
- Ovulation, 3-7, 12, 14-24, 33-5, 40-1, 63-8,
  - 202
- Ovulatory cycle, 3-5, 7, 14-15, 41, 195-200
- Ovum (*cf.* Follicle), 11, 30-7, 40, 64-8
- Oxygen consumption, 57, 211-19
- Oxytocin, 12-13
  
- Pacemaker, of chick amnion, 274-5
- Palmitic acid, 87, 92-9
  - clearance, 114
- p*-amino-hippurate (PAH), 287-92
- Pancreas, glucagon content of, 122
- Parathyroid, 197-201
- Pempidine, 264-6
- Penicillin (*cf.* antibiotics), 287
- Peristaltic action of gut, 269
- Perivitelline layer, 30-4

- Plumping, 63-73  
 Polyspermy, 42  
 Posterior vena cava, ligation of, 297-300  
 Potassium, 48, 63-73  
 Pregnenolone (*cf.* Hydroxysteroids)  
 Proctodaeum, 44-7, 103  
 Progesterone, 3, 7, 30, 37, 133-43  
 Prosthetic group (B<sub>12</sub> as), 88  
 Proteins  
   egg white, 63, 71, 136-42  
   seminal fluid, 48  
   synthesis in oviduct, 125-31  
   water-soluble magnum proteins (WSMP),  
     138-43  
   water-soluble oviduct proteins (WSOP),  
     133-43  
   yolk, 199-200  
 Protein requirements, 146-52, 171-80, 237  
 "Protein effect" 171-2  
 Puromycin, 129  
 Pyridostigmine, 254
- Radioactive isotopes (*cf.* Isotopes, radio-  
 active)  
 Renal function, 286-92  
 Reproductive tract (*cf.* Oviduct), 44-6  
 Reserpine, 113-9, 280-1  
 Resorption (of yolk), 19  
 Respiration, effect of vagotomy on, 302-10  
 Respiration chamber, 211-4  
 Respiratory diseases, susceptibility to, 212,  
   226, 243  
 Respiratory quotient, 215-8  
 Rete testis, 45  
 Ribonuclease, 130  
 Round folds (*cf.* Erectile structures)
- Secretion  
   accessory reproductive glands, 47-50  
   kidney tubules, 287-9  
   oviduct, 41, 125, 134  
   shell gland, 191-8  
 Semen, 39-43, 44-50, 52-9  
   collection, 46-7, 53-5  
   dilution, 44, 50, 54  
 Seminiferous tubule, 45, plate 11  
 Shell  
   formation, 69, 191, 199-202  
   electrolyte composition, 65  
   quality, 11  
   strength, 203-7  
   specific gravity, 206  
   thickness, 203, 205-7  
 Shell gland (Uterus), 3, 7, 11-13, 19, 41,  
   70, 191-8  
   blood supply, 181-8, plate 16  
   fluid, 73, 197  
   innervation, 12  
   thread in, 12, 197
- Shell-less eggs (*cf.* Eggs), 11-12, 197  
 Smooth muscle, of amnion, 274-8  
 Social Rank, 53  
 Sodium, 63-73  
 Sodium pentobarbital (*Nembutal*), 17, 306-9  
 Soft-shelled eggs (*cf.* Eggs), 12, 19, 81  
 Spectrophotometry (*cf.* Atomic absorption,  
   Ultraviolet)  
 Spermatogenesis, 15, 52, 59  
 Spermatozoa, 39-43, 44-50, 52-9  
   motility, 40, 52-6  
 Sperm-storage glands ("Sperm nests"),  
   39-43  
 Sphincter, renal portal, 289-91, 294, 300  
 Sphingomyelin, 95-7  
 Spinal cord, transection of, 304  
 Stearic acid, 87, 92, 99  
 Sterility (*cf.* Fertility), 52-6  
 Steroids (*cf.* Hormones, Hydroxysteroids),  
   23-36  
 Stocking density, 235, 243  
*Streptococcus faecalis*, 186  
 Stress, 56, 103-4, 109-10, 238, 282  
 Student's *t* test, 18, 194  
 Succinylcholine, 255  
 Sulphur (<sup>35</sup>S), 149, 164  
 Sulphydryl compounds, effect on sperma-  
   tozoa, 49  
 Systolic pressure, effect of *nor*-adrenaline  
   on, 120
- Temperature  
   body, 211-26  
     fatal, 225  
     regulation, 211-26, 228-9  
   environmental, 211-26, 228-33, 235-41,  
     242-5  
     critical, 221  
     fluctuating, 235-40  
 Tetraethyl ammonium, 287  
 Tetramethylammonium, 264  
 Testis, 44-6, 57  
 Testosterone (*cf.* Hydroxysteroids), 53,  
   133-41  
 Theca interna, 30-7  
 Thread, in uterus, 12, 197  
 Threonine, 156-8, 166  
 Thorax, resting position of, 304-8  
 Thymus, 182  
 Thyroid, 52, 59  
 Thyroprotein, 52-8  
 Thyrotrophic cells (of adenohypophysis), 53  
 Thyroxine, 58  
 Total body analysis, of newly-hatched  
   chicks, 63-70  
 Toxicity  
   ICI compound 33828, 21  
   insecticides, 258-9  
 Transport, ionic (*cf.* Active transport),  
   68-73

- Transport, yolk materials, 73, 99-100  
 Triglycerides, 87-100  
 Tritium (T) (*cf.* Isotopes)  
 Tryptophan, 155-68, 237  
 Tubocurarine, 250-3  
 Tubular cells  
     of nephron, 72  
     of shellgland, 193-4  
 Twinning planes, in shell crystals, 203, 207  
 Tyrosin, 59  
  
 Urea (and Uric acid), 50, 287-9  
 Ultraviolet absorption spectrometry, 23, 297  
 Ureter, 46  
     catheterisation, 286  
     exteriorisation, 147  
 Urine, 23, 146-8, 214, 271, 287.  
 Urodacum, 46-7  
 Utero-isthmus junction, 11  
 Uterovaginal junction, 39-41  
 Uterus (*cf.* Shellgland)  
  
 Vacuoles, fat, in embryo liver, 87, 96  
 Vagina, 39-43, 47-50, 139  
 Vagus, 261-6, 302-10  
 Vagotomy, bilateral cervical, 302-8  
 Valine, 156, 166-8  
 Vasculature  
     of shellgland, 191-8  
     of kidney, 286-92  
     of liver, 294-300  
 Vas deferens, 44-50  
 Vas efferens, 45  
 Vasopressin, 12-13  
  
 Ventilation, 211-21, 222-6, 235, 241-5  
 Ventilation (pulmonary), 219-23  
 Vesicles, in follicle, 32, 131, plate 13  
 Vitamins (*cf.* Ascorbic acid, thyroprotein, etc.)  
     A, 76-7, 83-5, 181-3  
     B<sub>12</sub>, 87-101, 181-4  
     vitamins and fertility, 57-9  
     supplementation of low-pigment diet, 79-80  
  
 Warburg apparatus, 253  
 Water, accumulation of in egg, 63-73  
 Water, consumption of, 211, 224  
 Water-soluble oviduct proteins, (*cf.* Proteins)  
 White (of egg) (*cf.* Egg)  
     composition, 63-4, 69-72  
 White, thick, 69-72  
 White body (*cf.* Erectile structures)  
  
 Xanthophylls (zeaxanthin, lutein), 75-83  
  
 Yolk  
     banding in, 83  
     lipid content, 87-101, 122  
     pigmentation, 75-85  
     resorption, 19  
     synthesis, 21, 24, 30, 34, 63-4, 68, 73, 113, 119-200  
 Yolk lipids, extraction of, 78  
 Yolk sac, 89-90, 99  
  
 Zeaxanthin (*cf.* Xanthophylls, Pigments), 75-8  
 Zona radiata, 30-4

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